ENDOCRINOLOGY

VOLUME 66 MARCH, 1960 NUMBER 3

Copyright © 1960 by the Endocrine Society

THYROID RESPONSE TO PARTIAL THYROIDECTOMY, THYROXINE AND 2,4 DINITROPHENOL IN RATS WITH HYPOTHALAMIC LESIONS[1,2]

SEYMOUR REICHLIN

Departments of Medicine and of Psychiatry, Washington University School of Medicine, St. Louis, Missouri

ABSTRACT

Neural regulation of thyrotrophic function of the anterior pituitary has been investigated in the rat. Using *in vivo* thyroid I^{131} release as a measure of thyroid activity, the effects of thyroxine, partial thyroidectomy and 2,4 dinitrophenol (DNP) have been studied in normal rats and in rats with hypothalamic lesions that had produced a reduction in baseline thyroid function. L-thyroxine injections, 15 μg. per day, inhibited thyroid activity in 22 operated and 8 control rats to an equal degree. Partial thyroidectomy was followed by functional activation of the remnant in 14 out of 15 rats with lesions and in all of 12 control animals; thyroid regeneration occurred in all control rats, but was blocked in 7 of 15 rats with lesions. DNP produced thyroid inhibition in all of 46 control animals. In 56 rats with lesions DNP was significantly less effective in causing thyroid inhibition than in normal rats and in 5 animals thyroid activity was increased after DNP administration.

These observations are interpreted to indicate that the anterior pituitary gland is itself responsive both to increased and to decreased blood thyroid hormone concentration, but that the "setting" at which the pituitary reacts is governed by a nervous system mechanism which involves the hypothalamus. It is concluded that DNP acts on more than one site in inhibiting pituitary thyrotropin release and that there is an important DNP-sensitive neural component in this system.

CLASSICAL observations indicate that the synthesis and discharge of pituitary thyrotrophin (TSH) is regulated by the thyroid hormone. More recently it has become apparent that TSH release is influenced also

Received May 29, 1959.

[1] These studies were supported by United States Public Health Service Grant B834 and by a grant from the Commonwealth Fund.

[2] This paper was presented in part at the June 1958 meeting of the Endocrine Society.

by hypothalamic structures (for references see 1) and by metabolically active substances such as 2,4 dinitrophenol (DNP) (2–4). The following experiments were done to determine if the hypothalamus is an essential part of the mechanism by which thyroxine and DNP suppress and low blood thyroid hormone levels stimulate TSH secretion. Rats with hypothalamic lesions and sham operated controls were utilized as test animals and thyroid I^{131} release rate was used as a measure of activation by TSH.

METHODS

Several groups of male rats with hypothalamic lesions and sham operated control animals described as series I, II, III, and IV in another study (1) were used in this experiment. These animals had moderate to marked destruction of the anterior and middle hypothalamus with consequent thyroid gland inhibition of varying degrees. Conditions of diet and housing were identical as were the techniques used for measurement of thyroid function. Thyroid hormone blood levels were lowered by subjecting the rats to removal of three-quarters of the thyroid gland by a previously described technique (2). The operative method used in the present study differed in that atropine premedication was not used. The effect of thryroxine and 2,4 dinitrophenol (DNP) on thyroid activity was determined as follows. An initial baseline I^{131} release curve was obtained beginning 24 hours after the intraperitoneal injection of 10 μc. of I^{131}. A second injection of I^{131} was then given, and thyroid radioactivity at 24 hours again measured. Immediately after this initial count drug administration was begun and a second curve measured. The slope of each curve was calculated by the method of least squares and the effect of the drug expressed as the percentage change in slope in the treated as compared with the control period. Sodium L-thyroxine,[3] 15 μg., was administered daily intraperitoneally in a physiological saline solution containing 0.01 N NaOH. DNP was twice crystallized from acetone and given in one of three ways.

Experiment 1 (*Series II*). DNP was added to ground laboratory diet[4] in a concentration of 0.1%. Both control rats and those with lesions were given this ration *ad libitum*.

Experiment 2 (*Series III*). Five mg. of DNP in 1 ml. of distilled water (brought to pH 7.4 with NaOH) were administered intraperitoneally every 8 hours.

Experiment 3 (*Series IV*). At the time of operation each animal with a lesion was paired with a sham-operated control. Daily food intake by the lesion-bearing animals was measured and an identical amount of food was then offered to the normal member of the pair. In certain pairs, rats with lesions ate more food than did the control animals. In each pair, however, the animal with the lesion ate the same amount or, in the case of those with hyperphagia, more than its pair. During the test period DNP in a concentration of 0.1% was added to the diet.

In addition to studies of thyroid function, the effect of DNP on rectal temperature and on metabolic rate was determined in rats from Series III, using techniques described previously (1).

RESULTS

1. *The Effect of Thyroxine Injections on Thyroid Function in Rats with Hypothalamic Lesions*

A total of 22 rats with lesions and 8 control animals of the series III group were injected once daily for six days with 15 μg. of L-thyroxine, be-

[3] Obtained through the kindness of Dr. L. D. Bechtol, Travenol Laboratories, Morton Grove, Illinois.

[4] Ralston Purina Laboratory Chow.

TABLE 1. THE EFFECT OF THYROXINE INJECTIONS (15 μG. L-THYROXINE DAILY) ON THYROIDAL I^{131} RELEASE RATE IN RATS WITH HYPOTHALAMIC LESIONS

Group	No. of animals	Thyroidal I^{131} release rate					
		Initial		After thyroxine		% inhibition	
		%/Day	±S.E.	%/Day	±S.E.	%	±S.E.
Control	8	19.2	1.3	2.8	0.7	85.6	3.1
Lesion							
A. With initial release rate below the lowest control level	12	8.7	0.8	1.8	0.4	79.7	4.8
B. With initial release rate within the control range	10	19.7	1.0	2.6	0.3	87.2	1.4

ginning immediately after the initial neck count, which was obtained 24 hours after I^{131} administration. I^{131} release rate for each rat was compared with baseline I^{131} release rate determined a week previously.

Animals with lesions were considered in 2 groups, those whose initial release rate fell within the initial release rate of the control animals, and those whose initial release rate was less than the lowest value in the control group. Thyroxine injection led to inhibition of I^{131} release rate in every rat to a highly significant degree (Table 1). The extent of inhibition was expressed as the percentage change in slope after thyroxine. Mean percentage inhibition in control animals and in animals with lesions whose initial release rate was within the normal range was nearly identical (85.6 and 87.2% respectively). In rats with initially low release rate, the percentage inhibition was 79.7, but the difference between this group and the control group is not significant by statistical test ($t = 0.92$).

2. *Changes in I^{131} Release Rate after Partial Thyroidectomy*

A total of 12 control and 16 animals with hypothalamic lesions from series I and IV were available for study before and three weeks after the removal of three-quarters of the thyroid gland (Table 2). Initial release rate was significantly slower in animals with lesions than in the controls. After partial thyroidectomy each of the control animals showed an increase in I^{131} release rate, and each of the 8 rats with lesions in series I and 6 of the 7 in series IV also showed acceleration in the postoperative curve.

3. *Thyroid Regeneration in Animals with Lesions*

An estimate of remnant weight was made as in a previous study (5) by subtracting from the weight of the removed left lobe the weight of the portion removed from the right lobe at the time of thyroidectomy. This estimated remnant weight was compared with the weight of the portion of thyroid found at death (Table 2). In each control animal remnant weight increased, but more variable results were obtained in the animals with lesions. In series I 6 of 8 rats with lesions failed to show an increase in remnant weight after partial thyroidectomy. Three of these in fact had a thy-

TABLE 2. EFFECT OF PARTIAL THYROIDECTOMY ON THYROID I^{131} RELEASE RATE AND THYROID REGENERATION IN RATS WITH HYPOTHALAMIC LESIONS

Group	No. of rats	Body weight		Thyroid remnant weight			I¹³¹ release rate			
		At TTx†	At death	Left	Found	Change	Before TTx†	After TTx†	Change	
									% initial value	
		gm.	gm.	mg. ±S.E.	mg. ±S.E.	% ±S.E.	%/day ±S.E.	%/day ±S.E.	±S.E.	
Series I*										
Control	5	303	348	3.5 1.0	5.1 0.9	+ 80 40	18.5 2.5	32.9 4.1	89	34
Lesion without thyroid regeneration	6	305	301	3.2 0.2	2.7 0.4	− 19 11	13.2‡ 1.1	37.6‡ 7.2	167	27
Lesion with thyroid regeneration	2	304	306	1.7 —	3.1	+106 —	8.3 —	19.3 —	156	—
Series IV*										
Paired control	7	387	391	7.6 .4	9.9 1.3	+ 30 13	19.7 1.2	28.6 1.5	46.1	5.9
Lesion	7	408	402	4.9 .9	7.0 .7	+ 59 18	7.6 1.0	15.0 2.5	142.1	80.5

* These series refer to animals with anterior and middle hypothalamic damage described in a previous publication (1).

† TTx = Partial thyroidectomy.

‡ Mean values for release rate based on 4 animals. In the two others the rate was too rapid to be measured accurately, i.e., thyroid I^{131} content at 48 hrs. was 3% of the level at 24 hrs. in one case and in the second thyroid I^{131} was detectable at 24 hrs. but no longer at 48 hrs.

roid remnant weight at death which was less than that estimated to have been present at the time of partial thyroidectomy. In series II only one of the 7 rats with lesions failed to show compensatory hypertrophy after partial thyroidectomy. The individual rat without compensatory hypertrophy (initial remnant weight estimated at 7.8 mg., 7.1 mg. found at autopsy) had a small but definite increase in I^{131} release rate from 8.7 to 10.6% per day. Thyroid remnant weight rose from an estimated initial value of 8.5 to 13.6 mg. in the single rat that had no increase in I^{131} release rate.

4. *Effect of DNP on Thyroidal I^{131} Release in Normal Rats and Rats with Hypothalamic Lesions*

DNP effects on thyroid I^{131} release were studied in 46 control and 51 rats with lesions studied in three experiments (Table 3). In the first (series II), DNP was added to the food and the rats were allowed to eat *ad libitum*. All control animals had thyroid inhibition of at least 43.8% while in the lesion group 2 of 11 animals had a faster I^{131} release rate on DNP than before DNP and in 7 inhibition was less than 20%. Mean inhibition in animals with lesions was significantly less than in the control group.

In evaluating this experiment the possibility was considered that the difference in effect was due to variations in the intake of DNP-containing food. This question was investigated in two ways. In one experiment the

TABLE 3. EFFECT OF 2,4-DINITROPHENOL ON THYROIDAL I^{131} RELEASE RATE IN NORMAL RATS AND RATS WITH HYPOTHALAMIC LESIONS

Group	No. of animals	Initial release rate %/day ±S.E.		Percentage inhibition % ±S.E.	
Series II.*					
DNP fed ad-lib.					
Control	8	12.7	0.9	54.0	2.2
Lesion	11	9.7	0.8	15.3[6]	5.4
Series III.*					
DNP injected					
Control	10	19.5	1.1	49.6	6.8
Lesion	21	12.5	1.3	30.6[5]	4.9
(initial rate in normal range)	8	19.4	1.1	29.5[3]	5.5
(initial rate below control)	13	8.5	0.7	31.3[4]	7.4
Series IV.*					
DNP fed					
Control					
ad-lib. fed	9	22.6	0.9	83.9	2.1
paired	19	21.0	0.7	59.9[2]	2.4
Lesion	19	6.3[1]	0.4	27.7[1]	8.1

[1] Difference between lesion and both control groups significant ($P < .001$).
[2] Difference between paired and *ad-lib.* fed significant ($P < .001$).
[3] Difference between lesion and normal significant ($P < .05$).
[4] Difference between lesion and normal not significant ($P < 0.1 > 0.05$).
[5] Difference between all lesion animals and control significant ($P < .05$).
[6] Difference between lesion and control significant ($P < 0.001$).
* These series refer to animals with anterior and middle hypothalamic damage described in a previous publication (1).

DNP was given by injection, and in the other it was added to the food. In the latter experiment each of the paired control rats was offered an amount of DNP-containing diet equal to the quantity consumed by the rats with lesions. In all instances rats with lesions ingested amounts of diet equal to or more than the quantity taken by the controls. Thus, any reduced effectiveness of DNP in rats with lesions could not have arisen through the consumption of reduced amounts of the drug.

a. *Effect of DNP Injection (Series III).* DNP by injection produced smaller and less constant effects on I^{131} release than when administered in the diet, an effect probably due to rapid excretion of the drug. Despite large statistical variance in the control group, mean inhibition was significantly greater than that of animals with lesions. The blocking effect of lesions was more marked in some rats than in others. One animal had a more rapid I^{131} release rate while under treatment, and 4 other operated animals were inhibited to a lesser degree by DNP than the least inhibited of the control group.

b. *Control Paired with Operated Animals (Series IV).* In this experiment a control animal was paired with each lesion-bearing rat. Thyroid inhibition by DNP was again found to be significantly less in animals with lesions than in their controls. Differences were marked in certain individuals; for example, the least inhibited control rat was more affected than over half of the rats with lesions, and 3 of the brain-damaged rats displayed more rapid I^{131} release rate while getting DNP than when untreated.

It is interesting that the normal rats consuming DNP in the diet *ad libitum* had significantly more thyroid inhibition than did the normal rats paired with animals with hypothalamic lesions. This difference was due to the fact that food intake of brain-operated animals is reduced to a greater extent by the addition of DNP to the diet than is the intake of normal animals; the DNP intake of *ad libitum* fed animals is therefore higher. This phenomenon is similar to that observed by Kennedy (6) in hyperphagic rats given food adulterated with kaolin.

5. *Effect of DNP on Metabolic Rate and Body Temperature*

DNP apparently exerted a smaller effect on thyroid function in rats with hypothalamic lesions than in normal animals. Variations in dosage were excluded by injection and paired experiments. The possibility was next considered that DNP did not work as well because, in animals with lesions, the drug failed to produce the usual increases in metabolic rate or body temperature. A particular reason for considering this was the observation by Ramey and Goldstein that the effect of thyroxine on oxygen consumption was abolished in rats with hypothalamic lesions (7).

Measurement of oxygen consumption of series III rats before and one hour after intraperitoneal injection of 5 mg. of DNP (Table 4) failed to reveal any significant difference in metabolic responsiveness of rats with

TABLE 4. EFFECT OF INTRAPERITONEAL INJECTION OF 5.0 MG. OF 2,4-DINITROPHENOL ON OXYGEN CONSUMPTION IN NORMAL RATS AND RATS WITH HYPOTHALAMIC LESIONS

Group	No. of rats	Metabolic rate				Percent increase	
		Initial		1 hr. after injection			
		Mean ml./hr./100 gm.	S.E.	Mean ml./hr./100 gm.	S.E.	%	S.E.
Control	7	106.7	4.7	143.1	11.2	35.8	7.8
Lesion	20	110.7[1]	4.3	171.3[1]	13.1	61.4[1]	12.5

[1] Differences between rats with lesions and control animals not significant ($P > .05$).

lesions as compared with normal animals. In fact, the mean percentage increase in animals with lesions was greater than in normals, although this difference was not found to be significant statistically. The mean body temperature of rats with hypothalamic lesions was significantly higher than that of control animals (Table 5); nevertheless DNP in these rats produced an increase in body temperature which is not significantly different from that produced in control animals.

6. *Neuroanatomical Correlations*

a. *Response to Partial Thyroidectomy.* The 6 series I rats with blocked thyroid regeneration had lesions in the hypothalamus between the paraventricular nucleus and the median eminence (Fig. 1, 2). Two series I rats had thyroid regeneration: one had a very small lesion anterior to the paraventricular nucleus while the other had a lesion indistinguishable in position and size from those with regeneration "block." Series IV rats were described in detail in a previous paper (1). All animals had extensive damage to hypothalamic areas between the paraventricular nucleus and median eminence; 11 had moderate to marked damage to the hypophysial-portal vasculature. One rat is of particular interest because its entire hypothalamus was almost completely destroyed, including the primary portal plexus (Fig. 2). It grew poorly, had gonad atrophy, severe diabetes

TABLE 5. EFFECT OF INTRAPERITONEAL INJECTION OF 5.0 MG. 2,4-DINITROPHENOL ON BODY TEMPERATURE IN NORMAL RATS AND RATS WITH HYPOTHALAMIC LESIONS

Group	No. of animals	Body temperature					
		Initial		Increment at 1 hr.	Increment at 2 hrs.	Maximum increment	
		° C	S.E.	° C	° C	° C	S.E.
Control	8	36.9	0.1	1.0	0.9	1.1[2]	0.5
Lesion	20	37.6[1]	0.2	0.9	0.8	0.9[2,3]	0.5

[1] Difference between initial temperature in control and lesion group significant ($P < 0.02$).
[2] Difference between initial body temperature and maximum temperature after DNP significant ($P < .001$). Increment was calculated from individual responses to the drug.
[3] Difference between lesion and control not significant.

insipidus, extreme irritability, and low baseline levels of I^{131} release rate. Nevertheless, partial thyroidectomy was followed by increase in remnant size from 4.5 to 6.5 mg. and by increase in I^{131} release rate from 8.0 to 12.8% per day.

b. *Response to Thyroxine.* Thyroxine inhibition was studied in series III. The brains were available for examination in 16 rats; of these, 11 had large areas of destruction in the region between the paraventricular nucleus and the median eminence. In the others, damage to this region was slight.

c. *Response to Dinitrophenol.* Brains were available for study from series III rats and series IV rats. Damage varied from well localized lesions in the pre-optic area to almost complete hypothalamic destruction with extensive primary plexus damage. No correlations were apparent between the site or extent of damage and the degree of inhibition produced by DNP.

DISCUSSION

In the regulation of pituitary-thyroid activity TSH secretion is inhibited by high blood thyroxine concentrations and is accelerated when thyroxine

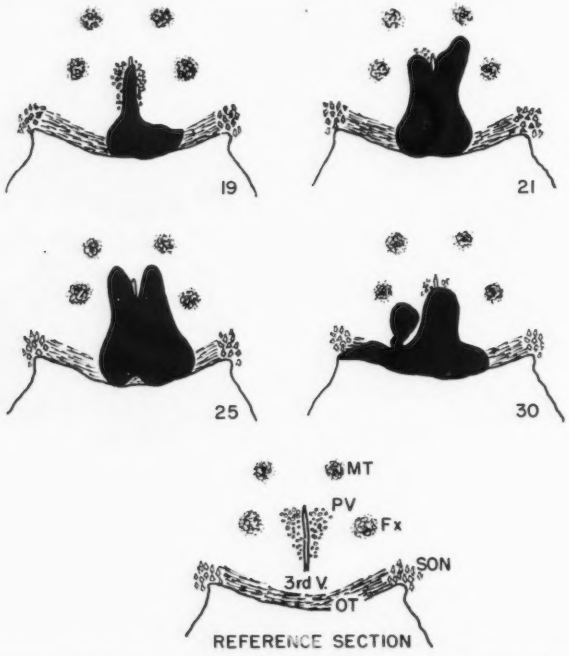


FIG. 1. Frontal sections of the brain at the level of the caudal limit of the paraventricular nucleus in four rats with thyroid "regeneration-block" are illustrated. The solid black area indicates the region of destruction. Landmarks in these sections are given in a diagram of the hypothalamus of a normal rat. Abbreviations: MT, mammillothalamic tract; Fx, fornix; Pv, paraventricular nucleus; 3rdV, third ventricle; OT, optic tract; SON, supraoptic nucleus.

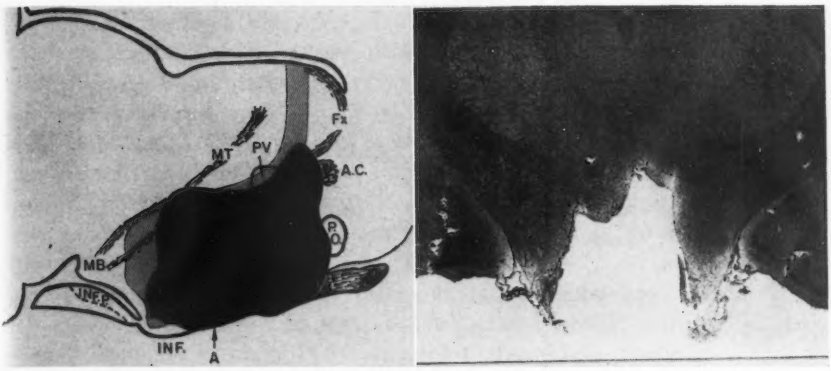


FIG. 2. The extent of hypothalamic damage in one rat is illustrated by a diagrammatic projection on a sagittal section and by a microphotograph of a coronal section at the level of maximum damage (level A). Almost complete hypothalamic destruction in this animal prevented neither thyroid regeneration nor increase in I^{131} release rate. Abbreviations: A.C., anterior commissure; Fx, fornix; Inf., infundibulum; Inf. P., Infundibular process; M.B., mammillary body; P.O., preoptic nucleus; P.V., paraventricular nucleus.

concentration falls. In this experiment the influence of the hypothalamus on the "feed-back" control of the pituitary-thyroid axis was investigated, using as experimental subjects rats with extensive hypothalamic lesions. These animals displayed marked abnormalities in thyroid function, as indicated by low I^{131} uptake and low rates of I^{131} release. Despite the destruction of thyroid-regulating neural structures, it was found that rats with lesions had qualitatively normal responses to changes in blood thyroid hormone levels. In such animals, as in normal controls, the injection of thyroxine was followed by marked inhibition of thyroid I^{131} release; after partial thyroidectomy rats with lesions and the control animals both developed an increase in the rate of I^{131} discharge from the remnant. Preservation of these characteristic responses in the face of massive hypothalamic damage permits the conclusion that the hypothalamus is not essential for the appropriate reactions of the pituitary to blood thyroxine concentration. A similar conclusion was reached by D'Angelo from studies in which plasma and pituitary TSH concentrations were determined by bioassay (8).

The findings in this study may be considered in the light of previous investigations of the role of the hypothalamus in the pituitary response to elevated and to lowered blood thyroid hormone levels. That thyroxine suppresses TSH activity without the necessary intervention of the hypothalamus or of the hypothalamic-pituitary neurovascular connection seems well established. In the rat (9) and rabbit (10) section of the pituitary stalk or transplantation of the pituitary to the anterior chamber of the eye (11, 12) causes a reduction in thyroid activity, but these animals show typical thyroid inhibition after the injection of thyroxine. A direct action of thyroxine

on the thyroid gland can be excluded as the cause of this effect (10). In rats, hypothalamic lesions prevent neither the normal depression of pituitary TSH content by triiodothyronine (8) nor the normal inhibition of thyroid I^{131} release which is produced by thyroxine (13). More direct evidence of a local effect of thyroxine on the anterior pituitary was obtained by von Euler and Holmgren (14). These authors demonstrated that thyroxine in amounts too small to cause thyroid inhibition when injected into the general circulation of the rabbit inhibited the thyroid when injected into the anterior pituitary.

The present experiments also indicate that the hypothalamus is not essential for the pituitary response to lowered blood thyroid hormone levels. Confirmatory data may be found in the literature although there is some degree of controversy. In rats with hypothalamic lesions propylthiouracil (PTU) administration is followed by the usual decline in pituitary TSH content (8), rise in serum TSH levels (8), degranulation of pituitary thyrotrophic cells (15) and increase in thyroid follicle cell height in some instances (15), a complex of events best explained as being due to mobilization of TSH. Furthermore, in the partially thyroidectomized rat the thyroid/serum iodide ratio (T/S ratio) rises, even in the presence of a hypothalamic lesion (16). In rats with hypothalamic lesions propylthiouracil administration is followed by the normal increase in the T/S ratio (17–19). Although the effects of PTU on iodide trapping in rats with lesions can be construed as evidence that TSH release is increased in these animals, interpretation of these findings is made difficult by the demonstration that PTU exerts direct effects on the thyroid gland and on its response to TSH (20, 21).

In this study hypothalamic lesions were found to produce inconstant effects on regeneration after partial thyroidectomy. In a large proportion of the animals regeneration was blocked, a finding similar to that of a previous study (16) and analogous to the propylthiouracil-goiter block reaction in rats with lesions first demonstrated by Greer (17–19) and confirmed by other authors (15). No anatomical feature distinguished those lesions which affected regeneration and function from those which affected function only. Much literature has accumulated about the significance of the dissociation between thyroid growth and thyroid functional response produced by hypothalamic lesions or by pituitary transplantation (4, 16–24); this question will be considered in detail in another publication (25).

In confirmation of the experiments of Wolff, Goldberg and their collaborators (2–4), dinitrophenol was found to inhibit thyroid gland activity in the normal rat. This characteristic response was significantly modified by hypothalamic lesions. In contrast to thyroxine, which is equally effective in normal and brain-damaged animals, DNP caused a smaller mean change in thyroid function in rats with hypothalamic lesions; in a few instances the usual effects of the drug were blocked completely by the lesion.

Before interpreting these results in relation to pituitary-thyroid axis regulation, their validity must be considered. A possibility that cannot be excluded absolutely is that modified DNP effects occur in rats with lesions because baseline thyroid function of these animals is abnormally low. Even with maximum inhibition the proportional change in I^{131} release might be less than that of control animals. A number of findings indicate that this possibility is unlikely and suggest that hypothalamic lesions do in some way interfere with DNP action on TSH release. In one of the experiments (Series III, Table 3) 8 of the operated rats had I^{131} release rates within the normal range, and in these animals inhibition by DNP injection was significantly less than normal. A total of 5 rats with lesions had an increase of I^{131} release rate while being treated with DNP; this kind of effect was never observed in normal animals. Finally, in series III rats that had a significantly reduced response to DNP injection thyroxine was found to have a normal inhibitory response. From these results the conclusion is drawn that DNP reduces thyroid activity in part at least through an action on a hypothalamic mechanism which influences pituitary thyrotrophic function. Although DNP responsiveness was reduced by the lesions, thyroid inhibition still occurred in most rats, even those with almost complete destruction of the hypothalamus. This indicates that DNP does not exert an effect exclusively on the diencephalon in producing changes in thyroid function.

Evidence has been presented in this paper that hypothalamic lesions reduce baseline thyroid activity but that the hormonal feedback regulation of the pituitary-thyroid axis may operate despite extensive hypothalamic damage. There is, moreover, evidence that in bringing about thyroid inhibition dinitrophenol acts at both diencephalic and pituitary sites. It would seem worthwhile to try to integrate these observations into a comprehensive hypothesis to explain the relationship between neural and the hormonal regulation of the pituitary-thyroid axis. Attempts of this kind have been made by a number of other authors (8–11, 14, 17–19, 26–33). It appears that the pituitary gland is autonomously responsive to changes in local thyroid hormone concentration regardless of the level of activity at which it is operating. In its reactions, it operates like a thermostatic controlling unit (31) and by analogy with thermostats and "glucostats" (34) may be considered as a "thyrostat." The hypothalamus, on the other hand, seems important in establishing the baseline level of thyrotrophic function. In rats with hypothalamic lesions resting thyroid activity is reduced (1, 8, 26) as are blood levels of TSH (8, 26) and protein bound iodine (26). Furthermore, hypothalamic damage or disruption of normal hypothalamic-pituitary connections is followed by abnormal sensitivity to thyroid hormone (10, 11, 13). As a mechanistic model it may be postulated that the setting of the pituitary "thyrostat" is governed by a hypothalamic mechanism which acts through the pituitary stalk. It is a reasonable supposition,

based on experimental observations, that through this hypothalamic mechanism the activity of the thyroid gland is homeostatically integrated with the nervous functions that control body temperature (1), energy expenditure, food seeking behavior, and heat balance.

Consideration must be given to the nature of the stimuli to which the hypothalamus is in turn sensitive in its regulation of the pituitary-thyroid axis. The observations of von Euler and Holmgren (14) of the effects of local micro-injection in rabbits indicated that the hypothalamus was not locally sensitive to thyroxine. Very recently however, Yamada and Greer (32) and Yamada (33), restudying the question in rats, have shown that intrahypothalamic injection of thyroxine is followed by thyroid inhibition, and by prevention of goiter formation after ingestion of propylthiouracil. In addition to local sensitivity to thyroxine, it is also possible that the hypothalamic control of pituitary-thyroid activity is responsive to changes in overall body heat or oxygen consumption. Such an interpretation would help explain how the neural temperature and heat regulating mechanisms are integrated with pituitary-thyroid interactions in response to changes in environmental temperature (35–37), heat loss (38), fever producing substances (4, 39), infections (40), or salicylate administration (41, 42). In this connection it should be pointed out that DNP, though possibly acting at hypothalamic and pituitary sites by intracellular effects shared with thyroxine (43), may also be effective because it raises cerebral oxygen consumption (44)—a response not shared by thyroxine (45). The elucidation of this question awaits further study.

Acknowledgment

The skilled technical assistance of Azmi Khazin, Mtanios Koussa, John Falch and Mrs. Christa Kindermann is gratefully acknowledged.

REFERENCES

1. Reichlin, S.: *Endocrinology* **66**: 340. 1960.
2. Wolff, J., L. Rubin and I. L. Chaikoff: *J. Pharmacol. & Exper. Therap.* **98**: 45. 1950.
3. Goldberg, R. C., J. Wolff and R. O. Greep: *Endocrinology* **56**: 560. 1955.
4. Goldberg, R. C., J. Wolff and R. O. Greep: *Endocrinology* **60**: 38. 1957.
5. Reichlin, S.: *Endocrinology* **62**: 463. 1958.
6. Kennedy, G. C.: *Ciba Colloquia on Endocrinology* **4**: 203. 1952.
7. Ramey, Estelle R. and M. S. Goldstein: *Fed. Proc.* **16**: 103. 1957.
8. D'Angelo, S. A.: *J. Endocrinol.* **17**: 286. 1958.
9. Uotila, U. U.: *Endocrinology* **26**: 129. 1940.
10. Brown-Grant, K., G. W. Harris and S. Reichlin: *J. Physiol.* **136**: 364. 1957.
11. Euler, C. von and B. Holmgren: *J. Physiol.* **131**: 137. 1956.
12. Khazin, A. and S. Reichlin: *Clinical Res.* **1**: 389. 1959.
13. Purves, H. D., N. E. Sirett and R. L. W. Averill: *Proc. Univ. Otago med. Sch.* **36**: 17. 1958.
14. Euler, C. von and B. Holmgren: *J. Physiol.* **131**: 125. 1956.
15. Bogdanove, E. M. and N. S. Halmi: *Endocrinology* **53**: 274. 1953.

16. Reichlin, S.: *Endocrinology* **60:** 567. 1957.
17. Greer, M. A.: *Proc. Soc. Exper. Biol. & Med.* **77:** 603. 1951.
18. Greer, M. A.: *J. Clin. Endocrinol. and Metab.* **12:** 1259. 1952.
19. Greer, M. A.: *Rec. Progr. in Hormone Res.* **13:** 67. 1957.
20. Halmi, N. S. and B. N. Spirtos: *Endocrinology* **55:** 613. 1954.
21. VanderLaan, W. P., and R. Caplan: *Endocrinology* **54:** 437. 1954.
22. Bogdanove, E. M., B. N. Spirtos and N. S. Halmi: *Endocrinology* **57:** 302. 1955.
23. Greer, M. A., R. O. Scow and C. Grobstein: *Proc. Soc. Exper. Biol. & Med.* **82:** 28. 1953.
24. Bogdanove, E. M. and J. N. Bogdanove: *Endocrinology* **61:** 52. 1957.
25. Reichlin, S.: *Endocrinology* (in press).
26. Florsheim, W. H.: *Endocrinology* **62:** 783. 1958.
27. Brown-Grant, K.: *Ciba Colloquia on Endocrinology* **10:** 97. 1957.
28. Ford, D. H. and J. Gross: *Endocrinology* **62:** 416. 1958.
29. Ford, D. H. and J. Gross: *Endocrinology* **63:** 549. 1958.
30. Goldberg, R. C., J. Wolff and R. O. Greep: *Endocrinology* **60:** 38. 1957.
31. Hoskins, R. G.: *J. Clin. Endocrinol.* **9:** 1429. 1949.
32. Yamada, T. and M. A. Greer: *Endocrinology* **64:** 559. 1959.
33. Yamada, T.: Presented at meeting of American Goiter Association, April, 1959.
34. Mayer, J.: *Physiol. Rev.* **33:** 472. 1953.
35. Dempsey, E. W. and E. B. Astwood: *Endocrinology* **32:** 509. 1943.
36. Brown-Grant, K., C. von Euler, G. W. Harris and S. Reichlin: *J. Physiol.* **126:** 1. 1954.
37. Woods, R. and L. D. Carlson: *Endocrinology* **59:** 323. 1956.
38. Reichlin, S. and Z. H. Lieberman: *Am. J. Physiol.* **195:** 659. 1958.
39. Gerwing, J., D. A. Long and R. Pitt Rivers: *J. Physiol.* **144:** 229. 1958.
40. Reichlin, S. and R. J. Glaser: *J. Exper. Med.* **107:** 219. 1958.
41. Austen, F. K., M. E. Rubini, W. H. Meroney and J. Wolff: *J. Clin. Invest.* **37:** 1131. 1958.
42. Wolff, J. and F. K. Austen: *J. Clin. Invest.* **37:** 1144. 1958.
43. Drysdale, G. R. and M. Cohen: *J. Biol. Chem.* **223:** 1574. 1958.
44. Messer, M.: *Australian J. Exper. Biol. & M. Sc.* **36:** 65. 1958.
45. Barker, S. B. and H. M. Klitgaard: *Am. J. Physiol.* **170:** 81. 1952.

THYROID FUNCTION, BODY TEMPERATURE REGULATION AND GROWTH IN RATS WITH HYPOTHALAMIC LESIONS[1,2]

SEYMOUR REICHLIN

Departments of Medicine and of Psychiatry, Washington University School of Medicine, St. Louis, Missouri

ABSTRACT

Electrolytic lesions of the hypothalamus were produced in 73 male rats. When compared with 64-sham-operated controls, certain rats with lesions had thyroid inhibition (decreased I^{131} uptake and release rate), persistent hyperthermia, diabetes insipidus, testicular atrophy and growth disturbance. Functional and neuroanatomical studies indicated that thyroid dysfunction, hyperthermia, diabetes insipidus and testicular atrophy were all independent consequences of localized brain injury. A temperature regulating center was localized to the pre-optic area; this partially overlapped the TSH regulating area which lies between the paraventricular nucleus and the median eminence. Gonad atrophy was caused by infundibular damage. As measured by rate of increase in body length (nose-tail) and by tibial length at death, growth was significantly impaired by certain hypothalamic lesions. No specific neural localization of the lesion responsible for poor growth could be made but it was noted that the most striking effects occurred with large median eminence lesions that had involved the primary portal plexus or stalk and had caused severe diabetes insipidus. India ink perfusion showed that the pituitaries were generally well vascularized. Hyperthermia and thyroid dysfunction were probably not the cause of the growth reduction since either or both of these abnormalities occurred in rats with normal growth rate.

LOCALIZED hypothalamic damage or separation of the pituitary gland from direct anatomical connection with the median eminence changes the function of the pituitary-thyroid axis in laboratory animals (1) and in man (2). Altered pituitary-thyroid function also occurs following electrical stimulation of the hypothalamus (3), or an emotionally "stressful" experience (4). Despite this evidence of central nervous system participation in the regulation of pituitary thyrotrophic hormone release the nature of the control mechanism and its relation to other visceral regulatory functions of the brain remain uncertain.

This paper is a report of studies carried out to elucidate the effect of hypothalamic lesions on thyroid function, and the relationship between

Received May 29, 1959.

[1] These studies were supported by United States Public Health Service Grant B834 and by a grant from the Commonwealth Fund.

[2] Presented in part at the June 1959 meeting of the Endocrine Society.

this effect and certain other disturbances which follow hypothalamic damage. These include hyperthermia, diabetes insipidus, hyperphagia, gonadal atrophy and growth failure. Also considered is the effect on pituitary blood supply of lesions which produce various endocrine abnormalities. The animals prepared for this study were used in subsequent experiments concerned with the role played by the hypothalamus in the response to alterations in blood thyroid hormone level and in the thyroid inhibitory response to dinitrophenol (5).

METHODS

Male rats of the Sprague-Dawley strain obtained from the Holtzman Company were used in these studies. They were housed singly in a temperature controlled room (26 ±1° C), exposed to constant periods of illumination and fed Purina Laboratory Chow and tap water *ad lib.* Hypothalamic lesions were produced with the aid of a Krieg-Johnson stereotaxic instrument utilizing a platinum electrode insulated to within 1.0 mm. of the end with epoxylite varnish. The platinum electrode was connected to the anode, and a subcutaneous hypodermic needle connected to the cathode. Four series of rats were prepared. In three of these bilaterally symmetrical lesions were placed 0.8 mm. anterior to bregma, 0.6 or 0.8 mm. from the midline, and 0.5 mm. above the floor of the skull. Current of 3 mA. for 30 seconds was used. Sham operated animals were prepared by inserting the electrode into the hypothalamus, but current was not passed. Unoperated controls were also studied. In the fourth experiment lesions were placed 0.8 mm. anterior to bregma, 0.5 mm. lateral to the midline, and 0.5 mm. above the base of the skull, and in some of the animals a second set of lesions were also placed in order to destroy the ventromedial nuclei as well (0.5 mm. posterior to bregma, 1.0 mm. lateral to the midline and at the base of the skull). Controls for this experiment were prepared by inserting the electrode only 2.0 mm. deep into the cortex. In the fourth experiment growth was specifically studied. Diet as a factor in causing growth disturbance in rats with lesions was evaluated as follows. At the time of the operation each animal with a lesion was paired with a sham-operated control. Daily food intake by the lesion-bearing animals was measured and an identical amount was then offered to the normal member of the pair. In certain pairs, rats with lesions ate more than did the control animal. In all instances, however, the food intake of rats with lesions was equal to or greater than the food taken by control animals. Nose to tail length at intervals after operation was measured in the anesthetized rat and the length of the tibiae were determined by vernier calipers from the cleaned bones after death.

Thyroid function was determined by measurement of the 24 hour I^{131} uptake and the rate of I^{131} release from the thyroid gland using an *in-vivo* technique (6). The counting of all animals was done under ether anesthesia because the irritability of many of the rats with lesions made difficult the usual handling methods. Neck counts were corrected for non-thyroidal neck radioactivity by subtracting values for neck radioactivity determined at comparable times in groups of six or seven totally thyroidectomized rats. Corrections for physical decay of radioiodine were made by comparing neck counts with an I^{131} standard. Serial radioactivity measurements were plotted on semi-logarithmic paper, and the rate of thyroid I^{131} release determined by calculating the line of best fit by the method of least squares.

Body temperature was measured with a thermistor thermometer[3] inserted 4 cm. into the anus for a 15 second period. In one experiment animals were exposed to the cold for

[3] Electro-Medical Engineering Company, Burbank, California.

three hours. They were moved to individual cages on the roof of the building on a moderately windy overcast day when the ambient temperature was 5° C. Exposure to elevated temperature was accomplished in one experiment by changing the setting of the animal room thermostat so that there was a gradual rise in room temperature over a 7 hour period from a level of 23.5° C to between 32.8 and 35° (depending on the position of the cage).

Metabolic rate was measured with a small spirometer. Corrections for temperature and barometric pressure were made and results expressed as ml. per hour per 100 gm. body weight. The rats were allowed to stay in the chamber for 30 minutes before the determination and the mean of two ten-minute runs were taken as the oxygen consumption of the animal.

At the time of death the rats in series I, III and IV were perfused via the carotid artery or abdominal aorta with 10 to 15 ml. of a suspension of one part of India ink (Higgins) to two parts of distilled water containing two drops of concentrated ammonium hydroxide per 100 ml. (7). Fixation of the skull in 10% formaldehyde was followed by decalcification in formic acid-formate solution (8), dehydration and embedding in celloidin. Prior to embedding, the skulls of rats in series III and IV were dissected carefully so as to expose the base of the brain and preserve the anatomical relationship between median eminence, pituitary stalk and pituitary gland. These structures could then be examined by means of a dissecting microscope and photographed. After embedding, coronal serial sections were cut at 40 or 120μ and stained with hematoxylin and eosin. The thyroid and adrenal glands were trimmed and weighed to the nearest 0.1 mg. on a torsion balance and the gonads to 0.01 gm. on a triple beam balance.

RESULTS

I. *The effect of hypothalamic lesions upon thyroid I^{131} uptake and release*

Data are available from a total of 137 rats (64 control, 73 lesion) prepared and studied in 4 series (Table 1). As measured by I^{131} uptake and release, thyroid function was significantly less in animals with lesions than

TABLE 1. THE EFFECT OF HYPOTHALAMIC LESIONS ON BODY WEIGHT, 24-HOUR THYROID I^{131} UPTAKE, AND THE RATE OF THYROIDAL RELEASE OF I^{131} IN RATS

Group	No. of animals	Interval between lesion and observation, days	Body weight		Thyroid I^{131} uptake		Thyroid I^{131} release rate	
			Initial, gm.	Final, gm.	% Administered dose		% per day	
					%	±S.E.	%/day	±S.E.
I Control	6	31	239	326	3.1	0.7	18.4	1.6
Lesion (anterior)	11	31	238	308	1.3	0.8	12.6	1.2
II Control	9	52	240	266	1.6	0.2	13.2	0.9
Lesion (anterior)	12	52	228	287	1.1	0.1	9.4	0.7
III Control	10	23	252	333	8.9	0.3	19.5	1.1
Lesion (anterior)	22	23	239	270	6.2	1.0	11.7	1.4
IV Control (*ad-lib* fed)	11	16	220	309	10.1	1.5	22.6	0.9
Lesion (anterior)	17	23	206	273	4.2	0.7	6.3	0.5
Control (paired)	17	23	205	305	9.8	0.2	20.1	1.4
Lesion (anterior and middle)	11	23	194	328	1.9	0.3	5.1	0.8
Control (paired)	11	23	195	320	10.6	0.7	19.9	0.5
Statistical evaluation (P values)								
I Lesion vs. Control					<.001		<.02	
II Lesion vs. Control					<.05		<.01	
III Lesion vs. Control					<.01		<.001	
IV Anterior Lesion vs. Paired Control					<.001		<.001	
Combined Lesion vs. Paired Control					<.001		<.001	
Anterior Lesion vs. Combined Lesion					<.02		>.1	

in sham operated or normal control animals. The degree of thyroid inhibition was variable in individual rats and ranged from perfectly normal values for uptake and release to almost complete thyroid inhibition (I^{131} release rate approaching 0% per day, I^{131} uptake as low as 8% of the average control uptake). This variability was probably a consequence of the heterogeneity of size and position of the lesions (vide infra). Inhibition was more marked in animals with two sets of lesions (anterior and middle hypothalamus, Series IV) than in those with anterior lesions only (Series I-III).

II. *Hypothalamic lesions and body temperature*

The position of lesions which in the rat produce thyroid inhibition are in the medial anterior hypothalamus. Lesions of the anatomically close preoptic area lead to disturbed temperature regulation in cats, as shown by the classical studies of Ranson and his co-workers (9). It is important therefore to determine whether or not the rats with lesion-induced thyroid inhibition also have disturbed body temperature regulation.

Measurements were made of rectal temperature in 2 series of rats (Table 2). In Series III readings at 76, 77, and 80 days after production of the lesion were made; the average of these was taken as the rectal temperature of each individual rat. Mean rectal temperature for the lesion group was significantly higher than for the normals ($P < .001$). In series IV temperature was measured 2, 4 and 8 weeks after production of the lesion. Animals with double lesions had significantly higher body temperature (1.0 to

TABLE 2. BODY TEMPERATURE IN RATS WITH HYPOTHALAMIC LESIONS

Group	No. of rats	Body temperature			
		76 to 80 days post-lesion	14 days post-lesion	29 days post-lesion	56 days post-lesion
		° C ±S.E.	° C ±S.E.	° C ±S.E.	° C ±S.E.
Series III					
Lesion	22	37.9 0.1			
Control	8	37.3 0.1			
Series IV					
Single Lesion	16		38.7 0.2	39.3 0.2	38.8 0.2
Paired Control	16		38.7 0.1	38.2 0.1	38.5 0.1
Double Lesion	11		39.1 0.2	39.6 0.2	39.3 0.2
Paired Control	11		38.1 0.2	38.5 0.2	38.3 0.2
		Statistical evaluation (P values)			
Series III					
Lesion vs. Control		< .01			
Series IV					
Single Lesion vs. Control			1	< .001	> .10
Double Lesion vs. Control			< .001	< .01	< .01
Single vs. Double Lesion			< .1 > .05	> .3	< .1 > .05

1.1° C) at all time intervals. Animals with a single lesion had statistically significant mean body temperature elevation only at 4 weeks after the lesion.

A further examination was made of the temperature regulatory defect in rats with lesions from series III by exposing them to cold and to heat. Over a 3-hour period of cold exposure mean rectal temperature of the 8 normal animals rose progressively from 37.7 to 38.3° C. In only one normal rat was there a fall in body temperature and this was slight (−0.4° C). In contrast, 9 of the 27 rats with lesions suffered a fall of body temperature in the cold ranging from −.2 to −2.5° C.

Exposure to gradually increasing room temperature from 23.5° C to between 32.8 and 35.0° C over a seven-hour period raised body temperature in both normal and operated rats (Fig. 1). In control animals body temperature rose an average of 2.4° C in the face of a mean ambient temperature rise of 11.0° C. The body temperature of 10 of the rats with lesions was normal and their response to rising temperature was exactly comparable to the controls. In contrast, 14 initially febrile animals with lesions suffered a smaller change in body temperature but reached maximum temperature levels sooner than did normals. Paradoxically, 4 animals with lesions manifested a transient depression of body temperature (up to 0.8° C) during the warming period.

III. *Diabetes insipidus in rats with hypothalamic damage*

Diabetes insipidus of varied severity was produced in a number of rats with lesions. Water intake was measured accurately, however, only in

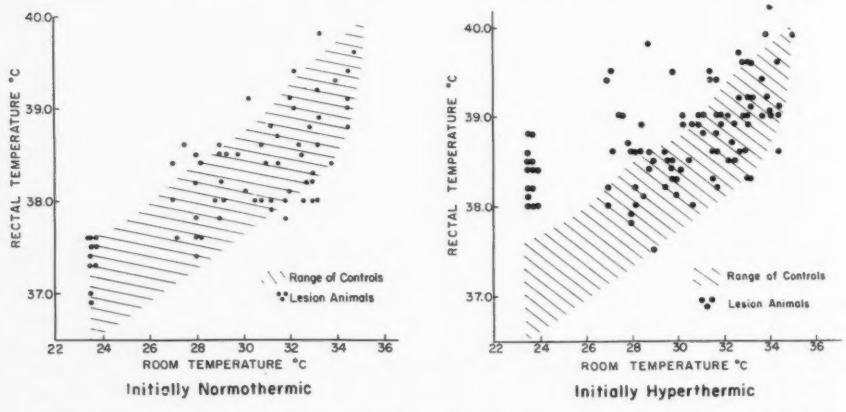


FIG. 1. The course of body temperature change in rats with hypothalamic lesions and in normal rats exposed to gradually increasing room temperature over a 7-hour period is depicted in this figure. Initially hyperthermic and normothermic rats with lesions are plotted separately. Each point represents a body temperature reading at a specific room temperature for a single rat (see text).

TABLE 3. METABOLIC RATE OF RATS WITH HYPOTHALAMIC LESIONS

Group	No. of animals	Metabolic rate ml. O_2/hr./100 gm. body weight	Standard error
Series III			
Control (*ad-lib* fed)	7	106.7	4.7
Lesion (anterior)	20	110.7	4.3
Series IV			
Control (*ad-lib* fed)	9	125.3	7.1
Lesion (anterior)	15	130.3	4.6
Control (paired)	15	132.6	4.1
Lesion (Combined anterior and middle hypothalamic)	9	108.2	5.0
Control (paired)	9	117.6	5.4

series IV. Mean water intake in all paired controls measured on three occasions was 28.5 ± 0.67 ml./day (range 22.9–36.6 ml./day). In the 26 animals with lesions 11 had mean daily water intake of less than 40 ml./day, 7 drank between 40 and 80 ml./day, and 8 drank more than 80 ml./day (maximum 149.0 ml./day).

IV. *Metabolic rate in rats with hypothalamic damage*

The occurrence of decreased thyroid activity in rats with lesions led to the consideration that such animals might exhibit abnormally low metabolic rate. Measurements of oxygen consumption were carried out therefore in series III and series IV animals (Table 3). Rats were tested in a random distribution. No significant abnormalities of metabolic rate were found.

V. *Effect of hypothalamic lesions on gonad and adrenal weight*

Series IV rats are here considered in detail in regard to adrenal and gonad weight (Table 4). Anterior hypothalamic damage only (as in the "single lesion group") had no effect on gonad weight in the group as a whole, and only one in 14 animals had a gonad weight less than the lowest control. Damage of an additional area, 1.3 mm. behind the first, led to a very striking decrease in mean gonad weight and 7 of the 8 rats so prepared had gonads that were $\frac{1}{8}$ to $\frac{1}{10}$ the size of those of their paired controls. Only one rat in this group had normal sized gonads.

TABLE 4. EFFECT OF HYPOTHALAMIC LESIONS ON GONAD AND ADRENAL WEIGHT

Group	No. of rats	Body weight		Adrenal weight		Adrenal wt. (mg./100 gm. body wt.)		Gonad wt.		Gonad wt. (gm./100 gm. body wt.)	
		Initial gm.	Final gm.	mg.	±S.E.		±S.E.	Gm.	±S.E.		±S.E.
Single Lesion	14	201	358	42.2	2.5	11.9	0.7	3.51	0.09	1.01	0.05
Paired Control	13	206	380	52.2	1.5	13.7	0.4	3.46	0.07	0.91	0.03
Combined Lesion	8	194	388	37.5	6.9	7.8	1.1	0.76	0.34	0.16	0.06
Paired Control	8	193	381	49.5	4.7	13.1	1.3	3.45	0.15	0.91	0.03
Statistical evaluation (P values)											
Single lesion vs. paired control				<.001		<.05		>.8		<.1>.05	
Combined lesion vs. paired control				>.2		<.01		<.001		<.001	
Single vs. combined lesion				>.4		<.01		<.001		<.001	

The adrenal glands also were affected by the lesions. This is true for animals with single lesions when compared both on an absolute basis and in relation to body weight, and was statistically significant in doubly operated animals when compared relative to body weight. When adrenal weights of individual pairs were compared, it was noted that marked adrenal atrophy had been produced in only three animals (26.1 vs. 48.8 mg., 23.6 vs. 61.8 mg., 8.6 vs. 55.5 mg.). These animals were in the doubly operated group and had severe damage to the pituitary stalk or primary plexus (vide infra). Two of the doubly operated animals had adrenal weights higher than the highest normal value. Both animals had severe diabetes insipidus; as pointed out by McCann and Brobeck (10), this is a common association.

VI. *Growth*

Growth was measured in a total of 28 rat pairs (Tables 5, 6, Fig. 2), using two 20-day reference periods, the first beginning 4 days and the second 56 days after operation. Tibial size was determined at death 114 to 117 days after production of the lesion. Data are not complete for several of the rat pairs.

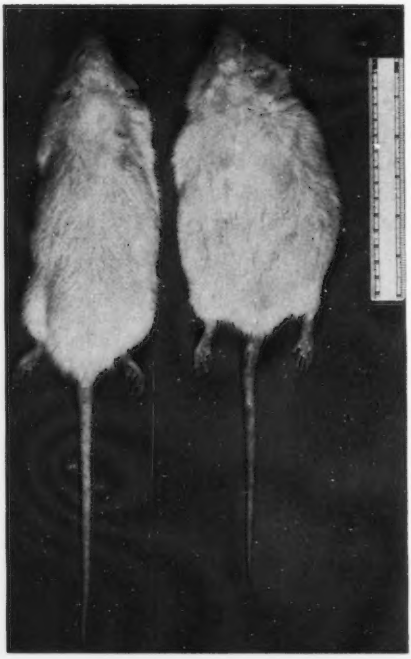


FIG. 2. Hyperphagic rat with anterior and middle hypothalamic damage (on the right) and sham operated control animal. To be noted is the smaller skull, shorter and more delicate tail, and shorter nose to tail and nose to rump length in the rat with the lesion, as well as the marked obesity.

Mean daily weight gain was significantly less in rats with anterior hypothalamic lesions than in their paired controls despite the fact that control animals actually ate slightly less food per day than did rats with lesions (Table 5). This difference is most clearly shown by calculating weight gain per 100 gm. of food intake.

More extensive hypothalamic damage, as produced by double lesions, was followed by a weight response different from that of rats with anterior lesions only. In part at least this was due to the associated hyperphagia. During the first period the animals gained in weight as rapidly as control animals, and in the second period even more rapidly. In the first period normal weight gain was maintained by abnormally high food consumption, but by the second period rats with double lesions

TABLE 5. EFFECT OF HYPOTHALAMIC LESIONS IN THE RAT ON LINEAR GROWTH, WEIGHT GAIN AND "FOOD UTILIZATION FOR WEIGHT GAIN"

Group	No. of rats	Body length						Mean daily weight gain				Mean daily food intake				Weight gained per 100 gm. eaten			
		28 days p.o.†		56 days p.o.		76 days p.o.		Period I 4–24 days p.o.		Period II 56–76 days p.o.		Period I 4–24 days p.o.		Period II 56–76 days p.o.		Period I 4–24 days p.o.		Period II 56–76 days p.o.	
		Cm.	±S.E.	Cm.	±S.E.	Cm.	±S.E.	Gm.	±S.E.	Gm.	±S.E.	Gm.	±S.E.	Gm.	±S.E.	Gm.	±S.E.	Gm.	±S.E.
Single Lesion	15	39.2	0.2	40.6	0.3	41.1	0.3	3.56	0.18	1.07	0.14	22.1	0.7	18.4	0.5	16.3	0.9	5.7	0.6
Paired Control	15	40.9	0.2	42.2	0.2	43.1	0.2	4.79	0.14	1.51	0.19	19.8	0.3	17.4	0.4	24.3	0.9	8.5	0.9
Combined Lesion	11*	37.4	0.3	37.8	0.2	38.1	0.3	5.61	0.51	2.74	0.47	27.4	1.8	22.4	1.6	20.5	1.4	11.6	1.2
Paired Control	11*	40.7	0.4	42.1	0.4	43.0	0.4	5.63	0.24	1.22	0.17	20.6	0.6	18.0	0.6	27.4	0.6	7.0	1.0
Statistical evaluation (P values)																			
Single Lesion vs. Control		<.001		<.001		<.001		<.001		<.1>.05		<.01		<.2>.1		<.02		<.02	
Combined Lesion vs. Control		<.001		<.001		<.001		>.9		<.01		<.01		<.02		<.001		<.01	
Single vs. Combined Lesion		<.001		<.001		<.001		<.001		<.001		<.01		<.01		<.001		<.001	

* Nine animals for length measurement.
† Postoperative.

TABLE 6. EFFECT OF HYPOTHALAMIC LESIONS ON TIBIAL LENGTH

Group	No. of rats	Body weight	Tibial length		
			Cm.	±	S.E.
Single Lesion	13	361	4.13		0.03
Paired Control	13	381	4.25		0.02
Double Lesion	8	455	3.95		0.03
Paired Control	8	394	4.29		0.01
Statistical evaluation (P values)					
Single lesion vs. control			<.001		
Double lesion vs. control			<.001		
Single vs. double lesion			<.001		

gained more weight relative to food intake than did normals.

Lesion-induced growth disturbance was more clearly demonstrated by measurements of nose-tail lengthening. Linear change was significantly reduced in rats with either single or double lesions, and in those with double lesions to a much greater degree. Growth was less in doubly operated animals despite greatly increased food intake and gain in body weight. With combined lesions, a number of animals became obese hypogonadal dwarfs (Fig. 2). As indicated by tibial size (Table 6), growth of the long bones was also affected by hypothalamic destruction. Deficient tibial lengthening was more marked in doubly operated animals.

The possibility was considered that an absorptive defect accounted for the abnormally low utilization of food for growth in rats with lesions. Fecal weight measured over a 3-day period was compared with the food intake in this interval. Normal animals and those with lesions all excreted a dry fecal mass equal to between 22.0% and 26.2% of the daily food intake.

VII. *Neuroanatomical correlations*

Series III and IV brains were studied in detail, the papers of Gurdjian (11) and Krieg (12) being used as a guide to the anatomy of the hypothalamus. The location of lesions in series I is mentioned elsewhere (5).

Hypophysial-portal vessels in the India-ink injected brains were examined under a stereoscopic microscope after the decalcified skull had been dissected away. Preparations of this type give a clear indication of the integrity or disorganization of the vasculature of the stalk and infundibulum. Forty brains were further studied in serial frontal sections and lesions plotted in characteristic planes obtained from tracings of a series of normal brains. In addition to outlining the general hypothalamic location involved, damage to the pituitary blood supply and to certain specified nuclei and tracts was also noted.

A. *Thyroid function and body temperature.* Characteristic of each of the 29 rats with thyroid inhibition was a midline lesion that involved the hypothalamus between the paraventricular nucleus and the anterior limit of the infundibulum. Added damage in the infundibular region caused an even greater reduction in thyroid activity; the lowest I^{131} uptakes were observed in 6 rats with almost complete destruction of the tuberal portion of the hypothalamus. Thyroid function was normal in 8 rats with lesions. One only had an extensive lesion between the paraventricular nucleus and the infundibulum while this region was either spared or only slightly involved in the others. Abnormal thyroid function can occur with a normal primary portal plexus or when the following paired structures are intact: fornix, mammillo-thalamic tract, suprachiasmatic, paraventricular, supraoptic, ventromedial, arcuate and mammillary nuclei. Normal thyroid function occurred in 8 individual rats despite the bilateral destruction of one or more of the following: fornix, anterior commissure, anterior periventricular

fibers and the supra-chiasmatic, paraventricular, pre-optic, arcuate and septal nuclei.

The lesions of Series III were smaller than those of Series IV and permitted more accurate separation of areas involved in temperature and thyroid regulation (Fig. 3). Five rats had fever and normal thyroid function; common to all was destruction of midline structures in the pre-optic area. In contrast, this area was uninvolved in 7 of the 8 rats with normal temperature and abnormal thyroid function. The temperature of the eighth rat was only 0.1° C above that of the highest of the control animals; it is likely that this animal was not significantly febrile. Three rats in series III

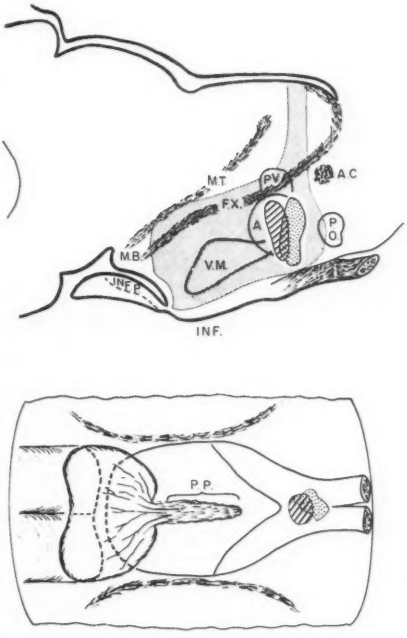


FIG. 3. Location of thyroid- and temperature-regulating areas in the rat hypothalamus based on studies of 12 rats in Series III. The stippled region illustrated in projections on a mid-sagittal section and on the ventral surface is the area of destruction common to all five of the rats who manifested chronic fever with normal thyroid I^{131} release rate. The hatched region is the area of destruction common to all of 7 rats whose thyroid I^{131} release rate was low but whose body temperature was normal. The two areas, though separate, partly overlap. Abbreviations: A.C., anterior commissure; Fx, fornix; Inf., infundibulum; Inf.P., infundibular process; M.B., mammillary body; M.T., mammillothalamic tract; P.O., pre-optic nucleus; P.P., primary plexus of hypophysial-portal vessels; P.V., paraventricular nucleus; V.M., ventromedial nucleus. The shaded area representes the outline of the third ventricle.

had both thyroid inhibition and fever, and in these animals pre-optic and paraventricular areas were simultaneously involved. It is thus apparent that the heat regulating area is separate from the thyroid regulating area, and that these regions are close together or overlapping.

In series IV 10 of 19 animals had chronic fever. Nine had extensive pre-optic damage. The tenth had no pre-optic damage, but had lateral hypothalamic damage in the tuberal plane, an area shown by Ranson to be the location of descending heat regulating fibers (9).

B. *Gonad atrophy.* Common to all rats with gonadal atrophy was destruction of the anterior portion of the infundibulum with slight or marked portal vessel damage.

C. *Diabetes insipidus.* Although some degree of diabetes insipidus occurred in 19 animals, in only 3 was there even slight direct damage to the main body of the supra-optic nucleus. All 19 had some degree of paraventricular damage. In the majority of instances diabetes insipidus was brought about by injury to the supraoptico-hypophysial tract just anterior to the infundibulum.

D. *Abnormal growth.* The brains of 24 rats from series IV were available for study. In this group 20 grew more slowly than their paired controls; of the 20, 9 had completely normal portal vessels, 6 had slight damage to the anterior portion of the primary plexus and 5 had extensive portal vessel or stalk damage. The most severely retarded growth was noted in rats with the greatest degree of primary plexus or stalk damage. The lesions used in this series were too large to permit any significant localization of growth-affecting structures; the most severe disturbance, however, occurred in rats with damage to the tuberal region. All rats with severe anterior infundibular damage had diabetes insipidus and gonad atrophy and it was this group that showed the most striking growth retardation.

DISCUSSION

In this study anterior hypothalamic lesions in the rat were found to reduce the activity of the thyroid gland, as measured by I^{131} uptake and release methods. Similar observations have been made by others in the rat (1, 13, 14, 15) and the dog (16). The effect of lesions on thyroid function is similar to that produced by section of the pituitary stalk in the rabbit (17). In common with stalk section, hypothalamic injury induces a degree of thyroid functional inhibition to values intermediate between those of normal and hypophysectomized animals. Mean I^{131} release rate in the four series of animals in this experiment was between 5.1 and 12.6% per day. In contrast, hypophysectomy reduces I^{131} release rate in every rat to less than 4% per day (unpublished observations). The change in thyroid function after brain damage was shown to be independent of non-specific effects on nutrition, for rats with lesions had less active thyroid glands than controls taking equal or smaller amounts of food. Non-specific changes due to disturbed pituitary blood supply were also excluded as a cause of thyroid inhibition by demonstrating intact hypophysial-portal vessels in many individual rats with much reduced I^{131} uptake and release rate.

The hypothalamic region involved in thyroid regulation appears from this study to lie in the anterior hypothalamus in the midline between the paraventricular nucleus and the infundibulum. Inhibitory effects are greater when larger, somewhat more posteriorly placed lesions are present. Thyroid regulating function of the hypothalamus has been localized to this general region in studies based on ablations in the rat (1, 14, 18, 19, 20, 21), dog (16), and guinea pig (22) and in stimulation experiments in the rabbit (3). More specific localization between paraventricular nucleus and median

eminence was suggested by Florsheim (14), Greer and Erwin (19) and by Ganong *et al.* (16). In the studies of Harris and Woods (3) the location of the electrode tip which gave thyroid activation upon stimulation was also in the anterior portion of the median eminence. Further evidence for specificity of a thyroid regulating area is the lack of effect on thyroid function of isolated damage to either the ventromedial (23) or paraventricular nuclei (24).

The localization of a thyroid regulating area to the anterior hypothalamus is of particular interest when considered in relation to temperature controlling functions. In this study, a temperature regulating area has been demonstrated in the rat in the pre-optic region between the anterior commissure and the optic chiasm in position corresponding to similarly described areas in the cat (9) and goat (25, 26). Neuroanatomical and physiologic data indicate that the thyroid regulating region is distinct from that for temperature regulation; they partially overlap, however. It is an attractive hypothesis that this general area serves to integrate short term heat loss, heat conservation and heat mobilization reactions with long term metabolic control by the pituitary-thyroid axis.

Consideration must be given to the mechanism by which the hypothalamus influences thyroid function. From classical observations on pituitary-thyroid interrelations and from studies described in detail by D'Angelo (1) it has appeared reasonable to conclude that the changes are due to altered thyrotrophic function of the anterior pituitary gland. Still uncertain, however, is the way in which these changes in pituitary function are produced. One hypothesis to explain the effect of hypothalamic lesions or of pituitary stalk section is that hypothalamic neurohumoral substances with TSH releasing properties reach the anterior pituitary by way of the hypophysial-portal vessels (17). Controversy surrounds the question of a neurohypophysial origin for this material in association with the antidiuretic hormone. It has been reported that median eminence extracts or Pitressin cause thyroid activation in the rat (27, 28, 29), dog (30) and rabbit (31), and in rabbits electrical stimulation of the anterior hypothalamus in a region close to the supra-optic hypophysial tract causes TSH release (3). In the present study, however, thyroid inhibition occurred whether or not diabetes insipidus was present after hypothalamic injury. Further evidence that the posterior lobe is not involved in thyroid regulation is the demonstration that Pitressin in amounts up to 2000 mU. daily or oxytocin in amounts up to 932 mU. per day have been found to exert no effect on thyroid I^{131} release in the rat (6, 32).

In this experiment rats with hypothalamic lesions were found to grow less well than normal animals. These animals utilized food poorly for weight gain, and in many instances had markedly reduced linear and long bone growth despite adequate food intake. This observation supports with more systematically gathered data the impressions previously gained by Bogda-

nove and Lipner (33) and by Kennedy (34) in experiments on the rat. The major question raised by these findings is whether or not they are evidence of a specific hypothalamic function for the regulation of growth hormone. This question cannot be answered with certainty because of the other visceral disturbances that follow hypothalamic destruction. In addition to any specific effects on growth hormone regulation, indirect effects on growth may be exerted through lesion-induced thyroid inhibition, gonadal atrophy, adrenal cortical dysfunction, chronic fever, diabetes insipidus or disturbed pituitary blood supply. Moreover, these abnormalities may exert an additive effect on growth. It is not likely that reduced thyroid function was the major cause of poor growth in these animals, since the occurrence of slow I^{131} release rate and impaired growth was not significantly correlated. Gonadal deficiency can also be excluded as the cause of poor growth in these animals by reference to Scow's studies; in male Holtzman rats slowing of growth was not apparent for at least 28 days after gonadectomy and even after 56 days the difference from the normal was only 14% (35). Adrenal cortical dysfunction is a possible cause of growth disturbance after production of hypothalamic lesions. Median eminence destruction interferes with ACTH release (36) and adrenal insufficiency is followed by poor growth (37). In view of the substantial size of the adrenals found in these animals (Table 4) adrenal insufficiency is probably not a significant factor in these experiments.

Abnormal blood supply as a cause of pituitary growth hormone disturbance requires close consideration. In the material studied only slight growth failure occurred in animals with intact hypophysial-portal vessels. On the other hand, considerable damage to portal vessels or the pituitary stalk was observed in all animals with substantial growth reduction, although the pituitary proper showed excellent vascularization after India-ink perfusion. Gross circulatory impairment of the pituitary was not present in these animals but the changes in the primary portal plexus may have interfered with pituitary blood flow rate or with specific neurohumoral transfer.

With regard to the relationship between neurohypophysial function and growth little is known. Del Vecchio *et al.* (38) indicate that large doses of Pitressin cause an increase in tibial cartilage width in rats, and give reasons for concluding that this is due to a specific effect on growth hormone release. In the present experiment a fair correlation existed between the severity of diabetes insipidus and of growth disturbance; it would appear reasonable to investigate further the relation between supraoptico-hypophysial function and growth. Animals with severe diabetes insipidus uncontrolled by replacement therapy are under considerable stress, as indicated by adrenal gland hypertrophy and poor general appearance. These non-specific factors must also be considered in relation to growth disturbance. There is at present no direct evidence that growth hormone secretion is regulated by the

hypothalamus. In a suggestive study (39) rats with hypothalamic lesions developed insulin sensitivity, an abnormality corrected by the administration of growth hormone but not by adrenal steroids. More definitive proof that the growth abnormalities seen in rats with extensive lesions of the hypothalamus is due to specific growth hormone deficiency will require further investigation.

Acknowledgments

The skilled technical assistance of John Falch, Azmi Khazin and Mrs. Mary Ann Davies is gratefully acknowledged. Valuable help was also given by the Department of Illustration, Washington University School of Medicine.

REFERENCES

1. D'Angelo, S. A.: *J. Endocrinol.* **17:** 286. 1958.
2. McCullagh, E. P., M. Clamen and W. J. Gardner: *J. Clin. Endocrinol.* **17:** 1277. 1957.
3. Harris, G. W. and J. W. Woods: *J. Physiol.* **143:** 246. 1958.
4. Brown-Grant, K., G. W. Harris and S. Reichlin: *J. Physiol.* **126:** 29. 1954.
5. Reichlin, S.: *Endocrinology* **66:** 327. 1960.
6. Reichlin, S.: *Endocrinology* **60:** 470. 1957.
7. Harris, G. W.: *J. Physiol.* **111:** 347. 1950.
8. Engelbreth-Holm, J. and C. M. Plum: *J. Path. & Bact.* **63:** 751. 1951.
9. Ranson, S. W.: *Psychosomatic Med.* **1:** 92. 1939.
10. McCann, S. M. and Brobeck, J. R.: *Proc. Soc. Exper. Biol. & Med.* **87:** 318. 1954.
11. Gurdjian, E. S.: *J. Comp. Neur.* **43:** 1. 1927.
12. Kreig, W. J. S.: *J. Comp. Neur.* **55:** 19. 1932.
13. D'Angelo, S. A. and R. E. Traum: *Endocrinology* **59:** 593. 1956.
14. Florsheim, W. H.: *Endocrinology* **62:** 783. 1958.
15. Purves, H. D., N. E. Siretт and R. L. W. Averill: *Proc. Univ. Otago med. Sch.* **36:** 17. 1958.
16. Ganong, W. F., D. S. Fredrickson and D. M. Hume: *Endocrinology* **57:** 355. 1955.
17. Brown-Grant, K., G. W. Harris and S. Reichlin: *J. Physiol.* **136:** 364. 1957.
18. Bogdanove, E. M. and N. S. Halmi: *Endocrinology* **53:** 274. 1953.
19. Greer, M. A. and H. L. Erwin: *Endocrinology* **58:** 665. 1956.
20. Bogdanove, E. M.: *Endocrinology* **60:** 689. 1957.
21. Greer, M. A.: *Rec. Progr. in Hormone Res.* **13:** 67. 1957.
22. Bogdanove, E. M. and S. A. D'Angelo: *Endocrinology* **64:** 53. 1959.
23. Van Dyke, D. C., M. E. Simpson, S. Lepkovsky, A. A. Koneff and J. R. Brobeck: *Proc. Soc. Exper. Biol. & Med.* **95:** 1. 1957.
24. Olivecrona, H.: *Acta physiol. scand.* **40:** suppl. 136. 1957.
25. Andersson, B., R. Grant and S. Larsson: *Acta physiol. scand.* **37:** 261. 1956.
26. Andersson, B.: *Acta physiol. scand.* **41:** 90. 1957.
27. Arimura, A., Y. Takagi and T. Ueno: *Jap. J. Physiol.* **6:** 284. 1956.
28. Shibusawa, K., S. Saito, K. Nishi, T. Yamamoto, K. Tomizawa and C. Abe: *Endocr. jap.* **3:** 116. 1956.
29. Dubreuil, R. and L. Martini quoted by G. W. Harris and J. W. Woods: *J. Physiol.* **143:** 246. 1958.
30. Fraja, A. and L. Martini: *Arch. internat. pharm. dyn., Gand.* **93:** 167. 1953.
31. Bottari, P. M.: *Ciba Foundation Colloquia on Endocrinology* **11** p. 52, 1957. Little, Brown and Company, Boston.

32. Crosson, J. A., J. Falch and S. Reichlin: Abstract of Endocrine Society. p. 70, 1959.
33. Bogdanove, E. M. and H. J. Lipner: *Proc. Soc. Exper. Biol. & Med.* **81:** 410 1952.
34. Kennedy, G. C.: *J. Endocrinol.* **16:** 9. 1957.
35. Scow, R. W.: *Endocrinology* **51:** 42. 1952.
36. McCann, S. M. and J. R. Brobeck: *Proc. Soc. Exper. Biol. & Med.* **87:** 318. 1954.
37. Kuizenga, M. H., J. W. Nelson and D. J. Ingle: *Proc. Staff Meet. Mayo Clin.* **15:** 324. 1940.
38. Del Vecchio, A., E. Genovese and L. Martini: *Proc. Soc. Exper. Biol. & Med.* **98:** 641. 1958.
39. Spirtos, B. N., W. R. Ingram, E. M. Bogdanove and N. S. Halmi: *J. Clin. Endocrinol. and Metab.* **14:** 790. 1954.

DIPHENYLHYDANTOIN AND THE RELEASE OF ADRENOCORTICOTROPIC HORMONE IN THE ALBINO RAT[1]

D. D. BONNYCASTLE[2] AND A. J. BRADLEY[3]

Department of Pharmacology, Yale School of Medicine, New Haven, Connecticut

ABSTRACT

Rats, treated with diphenylhydantoin (Dilantin, 100 mg./kg.) fail to show the expected depletion of adrenal ascorbic acid following unilateral adrenalectomy, intravenous Vasopressin, subcutaneous epinephrine or intraperitoneal histamine. This blockade seems to occur in two stages, an initial phase presumably central in which the adrenal cortex maintains its ability to respond in a normal manner (by a depletion of ascorbic acid) to exogenous corticotropin; and later a second phase in which there is a reduction in the responsiveness of the adrenal itself to exogenous corticotropin.

THE therapeutic use of diphenylhydantoin (Dilantin) in humans has been shown to result in a marked decrease in the urinary excretion of 17-ketosteroids and glucocorticoids (Costa, Glaser and Bonnycastle (1)). Bray, Ely and Kelley (2) found that children treated with diphenylhydantoin for convulsive disorders exhibited a smaller increase in plasma 17-hydroxycorticosteroids in response to a test dose of 25 international units of corticotropin given intramuscularly, than did normal children, or epileptic children without treatment or when treated with some other anticonvulsive agent. The authors concluded that these observations suggested that chronic Dilantin therapy in the epileptic child produced a degree of adrenal cortical unresponsiveness.

The data presented in this paper describe the inhibitory action of diphenylhydantoin in rats upon the response of the pituitary-adrenal system to a number of stimuli known to cause a reduction of adrenal ascorbic acid.

Received June 22, 1959.

[1] Supported in part by a grant from The James Hudson Brown Memorial Fund of the School of Medicine, Yale University.

Appreciation is expressed to Doctor Colin White, Yale University, for statistical advice and to Dr. C. V. Winder, Parke, Davis and Company, for Dilantin; to Dr. R. K. Richards, Abbott Laboratories, for Trimethadione, Paramethadione, Epidon and Phenurone; to Mr. K. Erickson, Sandoz Pharmaceuticals, for Mesantoin, to Dr. Jewell, Ayerst Laboratories, for Primidone, and to Dr. Davis, Hoffmann-LaRoche Inc., for samples of Ro-2-3059 and Ro-2-4523.

[2] Present address: Department of Pharmacology, Seton Hall College of Medicine and Dentistry, Jersey City 4, New Jersey.

[3] Present address: Department of Ophthalmology, University of Michigan, Ann Arbor, Michigan.

On chronic administration the drug appears to lead to a decrease in the sensitivity of the adrenal cortex to exogenously administered ACTH.

MATERIALS AND METHODS

Male rats, Sprague-Dawley strain (150–250 gm.), in groups of ten or more of uniform weight, were the experimental animals. Adrenal ascorbic acid was determined by the method of Roe and Keuther (3), and adrenal cholesterol by the method of Sperry and Schonheimer (4).

Adrenal glands were removed, stripped of adhering tissue, and weighed to the nearest 0.01 mg. on a Roller-Smith torsion balance, extracted in grinding tubes containing 6% trichloroacetic acid. Aliquots of the supernatant after centrifugation were taken for ascorbic acid estimation, and the dried residues used for cholesterol determinations.

Aqueous diphenylhydantoin solutions (1%) were prepared every other day, a small quantity of alkali being added to give a clear solution having a pH 8.5–10 and kept under refrigeration. Injections of diphenylhydantoin 100 mg./kg. i.p. every twelve hours for a number of days were found to be effective in altering the response of the adrenals to standard testing procedures, while injections of saline at the same pH for the same time were without effect.

Tests of the functional integrity of the pituitary-adrenal system

1. *Unilateral adrenalectomy.* After administration of an anesthetic dose (5 mg./100 gm.) of pentobarbital, one adrenal gland, the control gland, was removed, and an hour allowed to elapse before removing the second gland. The normal animal with a functionally intact pituitary-adrenal system will respond to this procedure with a decrease in adrenal ascorbic acid.

2. *Substances which cause adrenal ascorbic acid depletion.* Among the many substances known to cause ascorbic acid depletion in intact normal animals, intravenous Vasopressin, intraperitoneal histamine and subcutaneous epinephrine have been used extensively as testing agents. In this study, these agents were administered by the routes indicated, and one hour later the adrenal glands were removed for analysis.

3. *The effect of corticotropin.* To determine whether effects had been produced directly upon the adrenal cortices, threshold doses of corticotropin were given intravenously, and one hour later the adrenal glands were taken for analysis.

Statistical analysis of the results was carried out applying Student's t test for significance (5). Data are presented in the form of bar graphs with the standard errors indicated.

RESULTS

(a) *The effect of treatment with diphenylhydantoin upon the response to unilateral adrenalectomy.* Animals treated with diphenylhydantoin (100 mg./kg.) twice daily for three weeks were subjected to unilateral adrenalectomy. In the control group there was a significant reduction in adrenal ascorbic acid in the second gland ($P < 0.001$), while no change occurred in the diphenylhydantoin treated group; also the adrenal ascorbic acid of the animals which received diphenylhydantoin was observed to be equal to that of the resting levels of the untreated animals.

A second group of animals treated with the same doses of diphenylhydantoin for a period of one week were subjected to the same procedure (Fig. 1). As in the previous experiment, no significant change occurred

in adrenal ascorbic acid of the treated group in contrast to the depletion in the control group.

A third group of animals was treated for two days (four doses) and then subjected to the same operative procedure. Again, the response to unilateral adrenalectomy in the treated group failed to occur (Fig. 2).

The effect of a single dose of 100 mg./kg. diphenylhydantoin administered one hour before subjecting the animals to unilateral adrenalectomy was examined, and while it was found that no significant reduction in

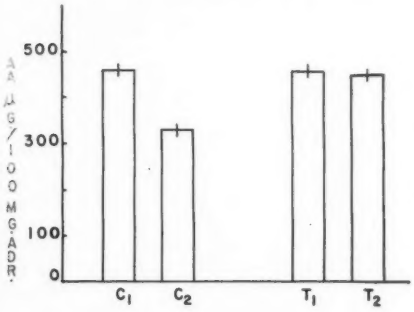


FIG. 1. The effect of unilateral adrenalectomy upon the adrenal ascorbic acid content of animals treated with diphenylhydantoin for one week. C_1 and C_2 = the ascorbic acid level of the 1st and 2nd adrenal glands of control animals; T_1 and T_2 the same levels in treated animals. The difference between C_1 and C_2 is significant, ($P < 0.001$) while the difference between T_1 and T_2 is not significant, nor do T_1 and T_2 differ from C_1.

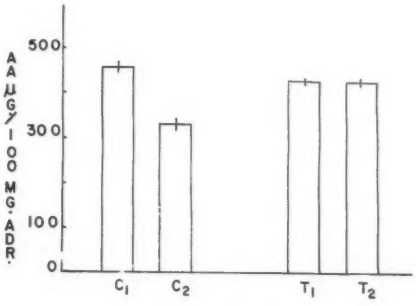


FIG. 2. The effect of unilateral adrenalectomy upon the adrenal ascorbic acid content of animals treated with diphenylhydantoin for two days. C_1 and C_2 = the ascorbic acid level in the first and second adrenal glands of the control group; T_1 and T_2 are the levels in the treated group. C_1 and C_2 differ significantly ($P < 0.001$) while the difference between T_1 and T_2 is not significant, nor do T_1 and T_2 differ from C_1.

ascorbic acid occurred in the second gland, the control adrenal ascorbic acid levels were lower than usual. However, if 24 hours were allowed to elapse after the administration of the drug, the resting control levels of ascorbic acid were found to be in the normal range, and no significant diminution was seen following unilateral adrenalectomy (Table 1).

The possibility of a delayed response in the diphenylhydantoin treated animals was studied by subjecting two-day treated animals to unilateral adrenalectomy and allowing 1, 2, 4 and 8 hours to elapse before removing the second adrenal. It was found that the ascorbic acid levels were the same in the first and second glands at each of the times examined.

A definite hypertrophy of the adrenal gland was regularly seen on chronic administration of diphenylhydantoin, as has been reported by others (6, 7, 8). In the present series of experiments it was found that after one week of treatment the gland size had increased some 30%, and after three weeks

TABLE 1. THE EFFECT OF UNILATERAL ADRENALECTOMY UPON THE ASCORBIC ACID LEVEL IN THE REMAINING ADRENAL EXAMINED ONE HOUR LATER, IN RATS TREATED WITH A SINGLE DOSE OF 100 MG./KG. DIPHENYLHYDANTOIN TWENTY-FOUR HOURS PREVIOUSLY

	Control animals		Treated animals	
	1st gland	2nd gland	1st gland	2nd gland
	433	320	542	494
	452	341	558	563
	467	315	470	400
	565	426	447	505
	508	402	506	390
	401	302	591	416
	420	341	431	404
	425	288	489	500
	490	348	521	482
$\bar{x}$	462	343	506	462
S.E.	±17.25	±15.09	±17.53	±20.22

of treatment by about 60% (see Table 2). A comparison of the dry weights of these enlarged glands, and those of the control glands show the enlargement to be the result of an increase in tissue, the water content of each group being the same, viz. 30–33%.

(b) *Duration of blockade after cessation of treatment with diphenylhydantoin.*

Groups of animals treated with diphenylhydantoin either for two days, or for a week, were examined at various intervals of time following the cessation of treatment to determine whether a return of the normal response to unilateral adrenalectomy occurred. After two days of treatment, some 75 hours were required before all the animals responded in a normal manner, while after one week's treatment between seven and ten days were required before all animals in the group responded.

(c) *Cholesterol content of the adrenal glands of diphenylhydantoin treated animals.* The cholesterol content of the adrenal glands was determined in a number of instances, however, the most complete series was carried out

TABLE 2. MEAN ADRENAL WEIGHTS OF RATS TREATED WITH DIPHENYLHYDANTOIN (100 MG./KG. INTRAPERITONEALLY B.I.D.) COMPARED WITH THOSE OF UNTREATED CONTROL ANIMALS OF THE SAME BODY WEIGHT

Group	Number of Doses	Number of animals	Right adrenal, mg.	Left adrenal, mg.
A				
Control				
125–50 gm.	—	10	10.20	11.36
Treated	1	9	11.12	11.62
Treated	4	8	13.96	14.20
Treated	14	7	13.81	14.36
B				
Control				
225–50 gm.	—	10	13.64	14.13
Treated	42	12	22.91	22.18

on animals after treatment for one week. The resting values were found to be 3.0–4.0 mg./100 mg. adrenal gland, which is within normal limits. No significant alterations were observed in the cholesterol content of the adrenals as a result of unilateral adrenalectomy of one hour's duration.

(d) *The response of diphenylhydantoin treated animals to agents known to cause adrenal ascorbic acid depletion.* Animals treated for two days in the usual manner were tested with the following agents: histamine phosphate 0.85 mg./100 gm. i.p., Vasopressin 0.08 unit/100 gm. i.v., and epinephrine

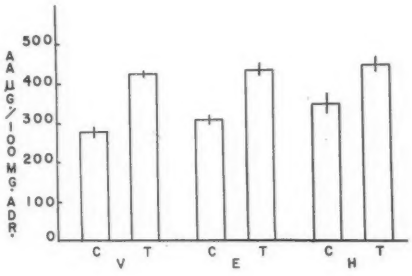


FIG. 3. The effect of Vasopressin 0.08 units/100 gm. i.v., epinephrine barbiturate 0.60 µU./100 gm. s.c., and histamine phosphate 0.85 mg./100 gm. i.p. upon the adrenal ascorbic acid content of control and diphenylhydantoin treated rats (two days). It is seen that the control animals all responded with a significant depletion of the ascorbic acid content, while the levels in the treated animals were unaffected.

bitartrate 60 µg./100 gm. s.c. Figure 3 presents the results obtained, and clearly indicates that the normally expected depletion of adrenal ascorbic acid following each of these substances failed to occur in the diphenylhydantoin treated group. Similar results were obtained with animals which had undergone one week of treatment.

(e) *The effect of exogenous corticotropin.* Large doses of (1 unit Armour) corticotropin were administered intraperitoneally to animals treated for one and three weeks with diphenylhydantoin, and while there was a significant reduction in adrenal ascorbic acid levels, this depletion was not as marked as that of the control animals.

Threshold intravenous doses of corticotropin (4 mU./100 gm.) were administered to groups of animals treated for either two days or one week with diphenylhydantoin. As shown in Figure 4, the same degree of ascorbic acid depletion was found in the untreated and two day treated groups. On the other hand, animals treated for one week prior to testing with 4 mU. ACTH/100 gm. showed adrenal ascorbic acid levels which did not differ significantly from control levels (Fig. 5). The difference between the values for these two groups and that of the untreated group given corticotropin is highly significant ($P = 0.01$). A similar finding was obtained when animals treated for one week with diphenylhydantoin were given 10 mU. of corticotropin/100 gm. intravenously (Fig. 6).

DISCUSSION

Rats treated with diphenylhydantoin (100 mg./kg.) either as a single dose or in repeated doses fail to exhibit the adrenal ascorbic acid depleting

effect of unilateral adrenalectomy, intravenous Vasopressin, subcutaneous epinephrine and intraperitoneal histamine (9). This interference with the normal response is probably dependent upon some alteration in pituitary-hypothalamic function, since early in the course of treatment the adrenal cortex remains fully responsive to exogenous corticotropin. However, if treatment with diphenylhydantoin is continued for a week or more a decrease in the responsiveness of the adrenal cortex itself to exogenous corti-

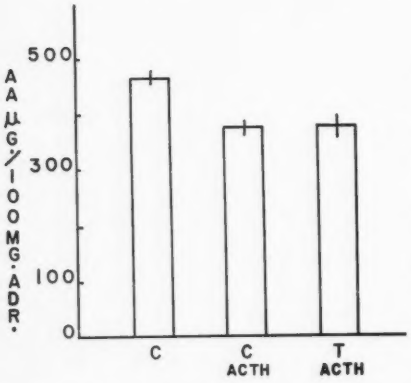


FIG. 4. The effect of corticotrophin 4 μU./100 gm. given intravenously, upon the adrenal ascorbic acid level of control and diphenylhydantoin treated rats (2 days). C = control resting ascorbic acid level of adrenal, C/ACTH and T/ACTH = the adrenal ascorbic acid levels of control and treated groups one hour after ACTH.

There is no significant difference between C/ACTH and T/ACTH, but both differ from C significantly (P approx. 0.01).

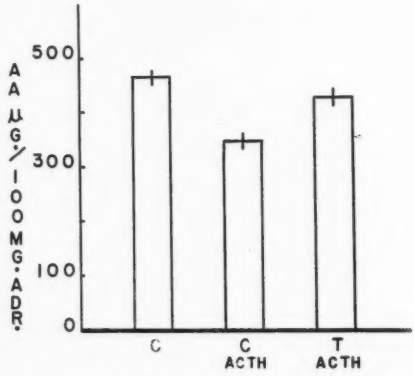


FIG. 5. The effect of 4 μU./100 gm. corticotrophin given intravenously upon the adrenal ascorbic acid level of control and diphenylhydantoin treated rats (1 week). C = control resting ascorbic acid level, C/ACTH and T/ACTH = the adrenal ascorbic acid level 1 hour after ACTH in untreated and treated rats, respectively. C/ACTH differs significantly from C and from T/ACTH (P = 0.01), while C and T/ACTH do not differ significantly.

cotropin becomes evident. The changes induced are not permanent, as far as has been determined, normal responsiveness of the adrenal cortex returning after treatment is stopped.

Of the substances other than the adrenal steroids reported to inhibit the normal response of the adrenal gland to a variety of noxious stimuli, morphine and amphenone have probably been the most extensively studied. In the case of morphine (10) the observations suggest that the blockade produced is in the pituitary-hypothalamic region or at some other site in the central nervous system; however, no studies have been reported with administration of the drug continued for more than one week. The adrenal ascorbic acid levels have been reported to be essentially normal but no reports concerning adrenal cholesterol levels are available. In amphenone in-

luced blockade (11) the resting levels of adrenal ascorbic acid are lower han normal and further depressed by exposure to cold, while the cholesterol values are greatly increased and are not apparently affected by the xposure to cold in a manner parallel to the adrenal ascorbic acid. These indings are in contrast to those of the diphenylhydantoin induced block, where both the cholesterol and ascorbic acid levels are normal and appear o be unaffected by any stress.

In seeking an explanation for the block caused by diphenylhydantoin, consideration must be given to the possibility of the persistence of an ele-

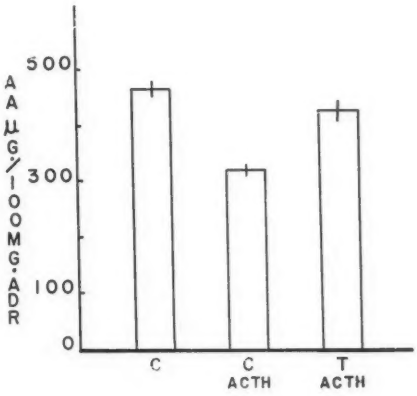


FIG. 6. The effect of 10 µU./100 gm. ACTH upon the adrenal ascorbic acid level of control rats and rats treated for one week with diphenylhydantoin. C control adrenal ascorbic acid resting level, C/ACTH and T/ACTH the adrenal ascorbic acid level 1 hour after ACTH. C/ACTH differs significantly from C and T/ACTH ($P < 0.001$) while C and T/ACTH do not differ significantly.

vated cortical hormone level in the blood of the treated animls for a number of hours after treatment. It is true that in the human studies (1) an increase in urinary corticoids was noted briefly after initiating a course of treatment with diphenylhydantoin. If these results are applicable to the rat the initial blockade might be considered to be steroid induced. The reduction in size of the thymi, the disappearance of most of the eosinophiles from the blood, and the adrenal hypertrophy of the treated rats lend additional support to the concept that the blockade is the result of an increased secretion of cortical hormones by the animals in response to drug treatment. However, the continued interference with the normal responsiveness to noxious stimuli and the subsequent refractoriness of the adrenals to exogenous ACTH should also be considered, in conjunction with the reduced corticoid excretion seen in humans as the administration of diphenylhydantoin is continued. On the other hand, adrenal hypertrophy is usually considered to be indicative of elevated ACTH levels, while adrenal atrophy is associated with excess steroid.

Measurements of the steroids of the blood in animals treated with dilantin have not yet been made, and it seems probable that the true explanation for the apparent blockade can only be settled when the results of such measurements become available. Whether there will be found to be any

great increase in blood steroid levels is questionable in view of the normal adrenal ascorbic acid levels (in contrast to the elevated ascorbic acid levels which we have observed after cortisol, i.e., an average of 620 μg./100 mg. adrenal in ten animals six hours after 10 mg. hydrocortisone subcutaneously), and also the observation of Bray *et al.* (2) that cortisol levels in the blood of normal children and epileptic children under chronic medication with diphenylhydantoin differed very slightly (normal 10 μg./100 cc.; epileptic group 11.2 μg./100 cc.). Probably studies of daily urinary steroid excretion coupled with the blood levels would be of greatest value in providing an understanding of these phenomena.

It appears from the work reported here that reliance upon changes in the adrenal ascorbic acid as an indication of a response to stress can be misleading. Some of these same questions raised above might be asked in relation to the failure of the adrenal ascorbic acid depleting response to one or more test stimuli following the administration of substances like morphine. Particular attention should be given to morphine for it is known (12) to cause adrenal hypertrophy on chronic medication as also does atropine (13). Morphine (10), atropine (14) and dibenzyline (15) have been reported to block the response of the adrenal to one or more test stimuli.

The change in the adrenal cortex that takes place after some days of treatment with diphenylhydantoin rendering it less susceptible to the effect of ACTH is of interest and requires further investigatoin. Attempts to mimic this effect by treating rats daily for one week with 5 units of ACTH, failed to produce an animal incapable of responding in the usual manner to the test procedure.

A number of other anticonvulsant drugs, 5-ethyl-3-methyl-5-phenylhydantoin (Mesantoin®); 5-ethyl-5-phenyl barbituric acid (phenobarbital); 5-phenyl-5-ethyl hexahydropyrimidine-4-6-dione (Primidone®); 3,5,5,-trimethyl-2-4-oxazolidine dione (Trimethadione®); 5-5-diphenyloxazolidine-2-4-dione (Epidon®); phenylacetylurea (Phenurone®); Ro-2-4523 and Ro-2-3059 were examined for their effect on the ascorbic acid depletion test; doses ranged from those reported to be anticonvulsant to those that produced signs of neurotoxicity (ataxia, sleepiness, etc.), and the dose repeated three or four times during two days, after which 10–12 hours was allowed to elapse before testing the ability of the adrenal to respond to unilateral adrenalectomy. In no instance was an indication of blockade observed.

REFERENCES

1. Costa, P. J., G. H. Glaser and D. D. Bonnycastle: *Arch. Neur. & Psych.* **74**: 88. 1955.
2. Bray, P. F., R. S. Ely and V. C. Kelley: *Arch. Neur. & Psych.* **72**: 583. 1954.
3. Roe, J. H. and C. A. Kuether: *J. Biol. Chem.* **147**: 399. 1943.
4. Schoenheimer, R. and W. M. Sperry: *J. Biol. Chem.* **106**: 745. 1934.

5. Fisher, R. A.: Statistical Methods for Research Workers. (10th Edition) Oliver and Boyd, 1946.
6. Woodbury, D. M.: *Recent Progress in Hormone Research* **10:** 65. 1954.
7. Woodbury, D. M.: *J. Pharmacol.* **105:** 96. 1952.
8. Staple, P. H.: *Lancet* **1:** 1074. 1951.
9. Bonnycastle, D. D. and A. J. Bradley: *Fed. Proc.* **15:** 403. 1956.
10. Munson, P. L. and F. N. Briggs: *Recent Progress in Hormone Research*, **11:** 83. 1955.
11. Hertz, R., W. W. Tullner, J. A. Schricker, F. G. Dhyse and L. F. Hallman: *Recent Progress in Hormone Research*, **11:** 119. 1955.
12. Selye, H.: *Arch. Internat. de pharm. et de therap.* **55:** 431. 1937.
13. Selye, H.: *Brit. J. exp. Path.* **17:** 234. 1936.
14. Guillemin, R.: *Endocrinology* **56:** 248. 1955.
15. Ohler, E. A. and R. W. Sevy: *Endocrinology* **59:** 347. 1956.

INDUCED AND SPONTANEOUS THYROID CANCER IN THE SYRIAN (GOLDEN) HAMSTER[1,2]

JOSEPH G. FORTNER, PHYLLIS A. GEORGE, AND STEPHEN S. STERNBERG

Experimental Oncology Section of the Division of Experimental Surgery of Sloan Kettering Institute, Andre and Bella Meyer Laboratories and the Department of Surgery, Memorial Center, New York, New York

ABSTRACT

Twenty-four or 12% of 204 hamsters fed an iodine deficient diet of rice and carrots developed thyroid cancer with metastases to lymph nodes or lungs. An additional 57 or 28% had probable cancer and 121 or 59% had thyroid hyperplasia. Histologically, the cancers were follicular adenocarcinomas. For the most part, they were so well differentiated as to preclude a definite diagnosis of cancer in the absence of metastases. The well differentiated, metastatic cancers are comparable to the "metastasizing struma" of man. The neoplastic changes in the thyroid gland were diffuse, suggesting TSH stimulation.

The findings were confirmed in a second experiment using hamsters from a different source. However, metastatic thyroid cancer was found only in females of this experiment. This could be a result of fortuitous selection or a manifestation of genetic differences.

In other experiments, spontaneous thyroid cancer was found in 8 or 1.5% of 523 hamsters surviving 181 days or longer on a diet of Purina Laboratory Chow. The cancers were of two histologic types, papillary and follicular adenocarcinoma and spindle cell carcinoma. Lymph node metastases were found. The spindle cell type was the more frequent and is of particular interest because of the presence of nuclear inclusions in the spindle cells. Areas of the thyroid not involved with cancer were normal histologically. This suggests an etiologic mechanism other than that of TSH stimulation.

THE experiments herein reported show that thyroid cancer develops in hamsters fed an iodine deficient diet consisting of rice and carrots. The tumors will be described and compared with the different types of thyroid cancer which occur spontaneously in this species. Most of the findings were incidentally observed in experiments designed to test human bile for carcinogenic activity (1–3).

MATERIALS AND METHODS

Induced Tumors: Animals used in the induced thyroid cancer studies were obtained from two commercial sources. In this first experiment, previously reported in part (4),

Received June 26, 1959.

[1] Supported by grants from the National Institutes of Health, U. S. Public Health Service.

[2] Read at the 1959 American Goiter Association Meeting.

TABLE 1. IODINE CONTENT (μG.%) OF RICE AND CARROT DIET

Date	Rice	Carrots
10/2/57	0.058	0.156
8/11/58	0.234	0.007
1/23/59	0.14	0.005
Mean	0.144	0.056

59 male and 60 female young adult hamsters were obtained from Tumblebrook Farms, Chesterton, New York. Prior to initiation of the experiment, the hamsters were fed Purina Laboratory Chow and water *ad libitum*, with supplements of sunflower seeds and carrots for 17 days. They were then grouped so that 2 of the same sex were housed together in a steel cage with a wire bottom. At this time, the animals weighed about 95 gm. The diet was changed so that it consisted only of an unmeasured amount of unpolished brown rice with approximately 1 gm. of fresh carrot per animal as a daily supplement. Tap water was given *ad libitum*.

A rice and carrot diet was chosen because of its ability to increase the susceptibility of rats to some liver carcinogens (5). At the time, we were evaluating the carcinogenicity of human bile and it seemed reasonable to utilize this diet for some of our studies. After a number of thyroid abnormalities had been observed, dietary iodine values were obtained. Three sample analyses gave consistently low values (Table 1). All iodine estimations were made by the method of Bodansky *et al.* (6).

Sixty-one of the animals available for examination were injected with human bile. Details concerning administration of the bile are not included, for the observed thyroid abnormalities were unaffected by it (Table 2). The animals were allowed to live out their life span or were sacrificed when moribund. Nineteen of the animals were autolysed or partially eaten when found and are omitted. Histologic studies were made on tissues stained with hematoxylin and eosin.

A second similar experiment was carried out on a larger group of animals obtained from a different breeder, namely, the Lakeview Hamster Colony, Newfield, New Jersey. Initial animal weights, maintenance and diet were essentially those described for the previous experiment except that pure bile salts or saline solution were administered to many animals. As shown in Table 3, this treatment did not appear to affect the development of thyroid abnormalities.

Spontaneous Cancer: Cancer of the thyroid gland was one of a variety of spontaneous

TABLE 2. THYROID PATHOLOGY IN 95 HAMSTERS FED A RICE AND CARROT DIET (EXPER. 1)

Group	Test material		Cancer with metastases				Probable cancer				Hyperplasia			
		Amount[1]	Males		Females		Males		Females		Males		Females	
	Type	Time[2] (days)	No.	Days[3]	No.	Days[3]	No.	Days[3]	No.	Days[3]	No.	Days[3]	No.	Days[3]
1	0	—	0	—	4	535	8	594	7	638	9	455	6	431
2	Bile (benign disease)	189 cc. / 263	0	—	5	508	6	568	4	627	8	352	6	382
3	Bile (malignant disease)	163 cc. / 248	0	—	0	—	7	316	7	523	8	296	10	282
Totals			0	—	9	520 (431–674)[4]	21	494 (101–712)[4]	18	591 (330–785)[4]	25	371 (36–725)[4]	22	349 (42–688)[4]

[1] Mean amount of bile injected.
[2] Mean number of days during which bile injected.
[3] Mean number of days during which the hamsters were fed a rice and carrot diet.
[4] Range of the values of No. 3.

TABLE 3. THYROID PATHOLOGY IN 204 HAMSTERS FED A RICE AND CARROT DIET (EXPER. II)

Group	Test material		Cancer with metastases				Probable cancer				Hyperplasia			
			Males		Females		Males		Females		Males		Females	
	Type	Amount[1] / Time[2] (days)	No.	Days[3]	No.	Days[3]	No.	Days[3]	No.	Days[3]	No.	Days[3]	No.	Days[3]
1	0	—	2	552	1	479	2	574	3	579	17	476	6	341
2	Saline	126 cc. / 236	1	594	2	625	4	558	5	522	9	389	11	448
3	Sodium Cholate	255.3 mg. / 235	0	—	0	—	3	487	6	266	10	479	10	473
4	Sodium Glycocholate	205.5 mg. / 183	1	801	0	—	5	637	5	415	4	613	6	456
5	Sodium Taurocholate	170.3 mg. / 188	2	571	2	618	3	550	4	413	4	384	6	494
6	Sodium Glycodesoxycholate	243 mg. / 299	5	598	3	504	5	347	4	561	8	304	10	420
7	Sodium Taurodesoxycholate	246 mg. / 247	0	—	5	427	7	482	1	594	10	384	10	383
Totals			11	603 (473–801)[4]	13	508 (218–768)[4]	29	473 (198–846)[4]	28	454 (161–728)[4]	62	430 (154–814)[4]	59	431 (160–828)[4,5]

[1] Mean amount of test material administered.
[2] Mean number of days during which materials given.
[3] Mean number of days during which the hamsters were fed a rice and carrot diet.
[4] Range for the values of no. 3.
[5] Two additional females dying after 65 and 115 days on the diet had normal thyroid glands.

tumors which were found in a group of 620 Syrian hamsters (7, 8). These animals were obtained as weanlings from the same source as that for the second experiment. Their diet consisted of Purina Laboratory Chow which according to the manufacturer has an iodine concentration of 200 μg. %. Water was given *ad libitum*.

The hamsters were subjected to a variety of experimental conditions. One hundred and sixty animals served as untreated controls; 120 received subcutaneously injected bile obtained from patients with cancer of the extrahepatic biliary tract; 340 hamsters were treated controls and were injected with bile obtained from patients with non-malignant biliary tract disease, sodium deoxycholate solution, distilled water, saline-bicarbonate solution, sesame oil or cholesterol in sesame oil. The animals were treated 3 times weekly for many months. They were allowed to live out their life span.

RESULTS

Induced Tumors: The appearance of hamsters fed the rice and carrot diet differed markedly from that characteristic of animals fed a balanced diet. The fur was thin and of light tan color. Mild scaling and long toenails were present. The skin was soft and velvet-like in texture. Body fat was minimal. In general, the viscera were small and the intestine translucent. The skeleton was extremely fragile. One hundred hamsters of the first experiment survived a mean of 441 days. Males survived a mean of 406 days (range: 36–725) and the females a mean of 474 days (range: 42–785). At death, body weight had decreased from an initial weight of about 95 gm. to a mean of about 55 gm. Study of the thyroid gland was possible in 95 of the 100 animals (Table 2).

Changes in the thyroid were progressive for the first 300 days. Grossly, the glands were red-brown and symmetrically enlarged (Fig. 1). With aging of the animal, nodularity was noted with increasing frequency. Microscopically, the earliest change was one of hyperplasia. This was characterized by a uniform increase in height of the follicular epithelium and a symmetrical enlargement of the follicles (cf. Figs. 2 and 3). With advancing age, a variation in the size and shape of the follicles and small papillary projections were noted. In localized areas, collections of follicles developed which were characterized by a lack of uniformity in size and shape. Some were markedly dilated and cystic with flattened epithelium; in others, papillary projections were prominent. Hyperchromatic nuclei were found in the cells of some follicles.

Nine or 18% of 49 females had thyroid cancer with metastases (Fig. 4). Pulmonary metastases were grossly apparent in 2. An additional 6 females were found to have metastases on routine microscopical examination of selected portions of each pulmonary lobe. In all instances, the metastases were located near the periphery of the lung. Metastases involving only the cervical lymph nodes were found in 1 animal. None of the males demonstrated thyroid cancer with metastases.

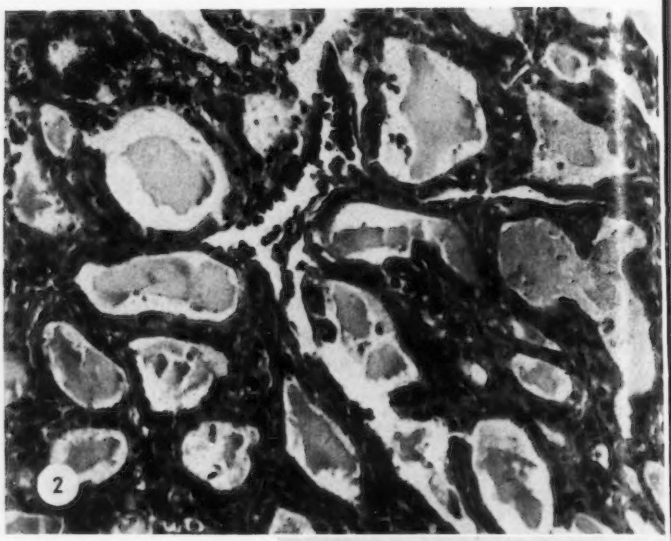
Histologically, neither the thyroid gland nor the metastases had that degree of atypia or anaplasia which is conventionally associated with cancerous changes (Figs. 5, 6). The changes were of the type usually seen in metastasizing struma. The metastases were all of the follicular type of carcinoma; no papillary formations were seen outside the thyroid gland. In the thyroid itself, papillary projections were seen but these were diminutive and of the type seen in hyperplasia. Because of this lack of anaplasia, a diagnosis of cancer was made only when metastases could be found. In the absence of metastases, comparable thyroidal changes were classified as probable cancer. Such lesions showed more cellular atypia than those called hyperplasia.

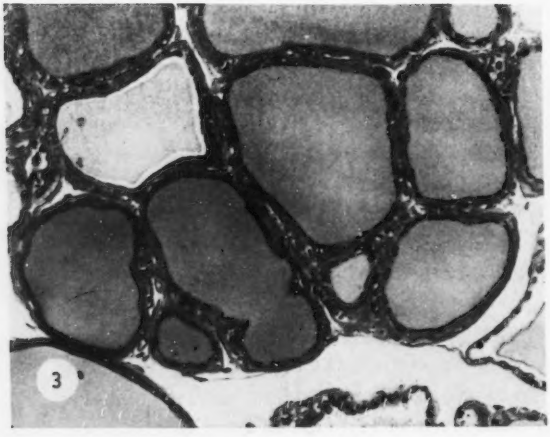
In the second experiment, 102 males survived from 154 to 846 days and 102 females survived from 64 to 828 days on the rice and carrot diet. An additional 17 animals, dying within the first 40 days on the diet, were not used. These animals did not adapt to the diet and seemed to die of starvation.

The thyroid glands were greatly enlarged. Fifty-one male hamsters surviving from 467 to 846 days on the experimental diet had thyroids with a mean weight of 160 mg. (range: 25–603 mg.). Forty-six female hamsters surviving from 231 to 828 days had thyroids with a mean weight of 219 mg. (range: 65 to 565 mg.). The normal thyroid gland of a hamster weighs about 7 mg.

In this experiment, thyroid cancer with metastases as well as other thyroid changes were found with about equal incidence in males and females (Table 3). Of the 204 hamsters, 12% had cancer with metastases;

1
CM


2


3

4
CM
1
2
3

TABLE 4. SPONTANEOUS THYROID CANCER

No.	Exper. Days*	Sex	Primary tumor		Spread		Treatment	
			Type	Location	Invasion	Metastases	Type	Amount
1	692 (23 mos.)	F	Papillary & follicular adenoca.	Entire gland; multinodular	Muscle	Lymph node	Untreated control	0
2	691 (23 mos.)	F	Papillary & follicular adenoca.	Left lobe 0.5 cm.	Muscle	0	Untreated control	0
3	1,000 (33 mos.)	M	Spindle cell carcinoma	Right lobe 0.5 cm.	0	0	5% glucose in saline/bicarbonate	187 cc.
4	358 (12 mos.)	F	Spindle cell carcinoma	Microscopic focus	0	0	1% cholesterol in sesame oil	21.5 cc.
5	635 (21 mos.)	F	Spindle cell carcinoma	Microscopic focus	0	0	Bile of patient without cancer	155 cc.
6	697 (23 mos.)	M	Spindle cell carcinoma	Microscopic focus	0	0	Bile of patient with cancer	110 cc.
7	846 (28 mos.)	M	Spindle cell carcinoma	Entire gland	Fat; lymphatics	Lymph nodes	Bile of patient without cancer	155 cc.
8	827 (27.5 mos.)	M	Spindle cell carcinoma	Entire gland	Muscle		Bile of patient without cancer	29 cc.

* Age of animal approximated by adding 30 to experimental days.

28% were thought to probably have cancer without metastases and 59% had hyperplasia. One per cent had histologically normal thyroid glands.

Histologically, the thyroid glands of these animals resembled those of the previous experiment (Fig. 7). However, follicular elements were less well differentiated in many animals, papillary projections were more prominent and hyperchromatic nuclei were more commonly seen (Fig. 8). Metastases were found in the cervical and/or mediastinal lymph nodes of 19 animals and in the lungs of 5 animals (Fig. 9).

No other sites of metastatic spread were observed. The metastases were follicular adenocarcinomas and resembled the primary tumor except for the absence of papillary projections.

Spontaneous Cancer: Five hundred and twenty-three of the 620 hamsters survived 181 days and longer. Eight or 1.5% had thyroid cancer (Table 4). Two of the cancers were low grade papillary and follicular adenocarcinomas (Fig. 10). They were found in females, 24 months of age, who were from the untreated group. Both tumors had local muscle invasion. One had metastases to the cervical lymph nodes. The metastatic lesions were histologically similar to the primary tumor. Psammoma bodies and inclusion bodies were not seen. One of the cancers involved

←

Figures 1, 2, 4–9 are induced tumors.

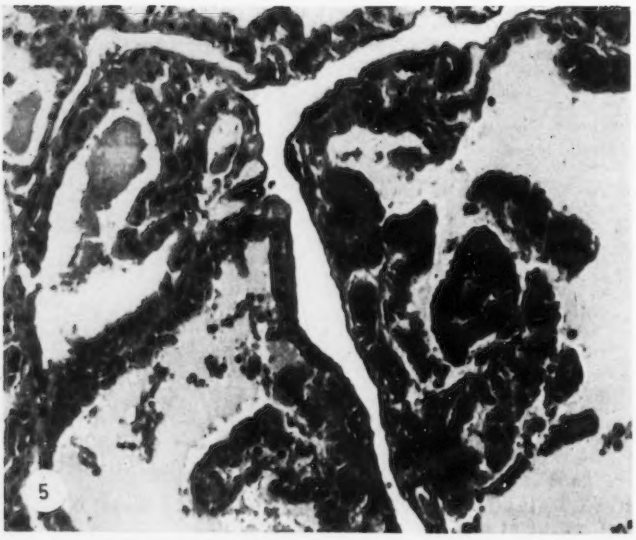
Figures 10–15 are spontaneous tumors.

FIG. 1. Posterior view of symmetrically enlarged thyroid gland of hamster fed the rice and carrot diet for 195 days.

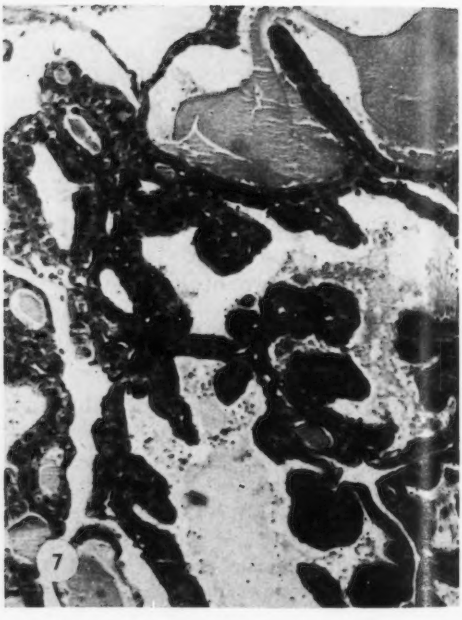
FIG. 2. Hyperplasia of thyroid gland. ×160.

FIG. 3. Normal thyroid gland. ×160.

FIG. 4. Posterior view of thyroid cancer in a female hamster fed the diet for 486 days. An enlarged paratracheal lymph node and multiple pulmonary metastases are evident.


5

6


7

only one lobe of the thyroid; the opposite lobe was normal in size and histologic appearance (Fig. 11).

Six of the 8 thyroid cancers were of the spindle cell type. They occurred in animals which had received a variety of substances: 1 was given 5% glucose in saline with bicarbonate; 1 received 1% cholesterol in sesame oil; 4 received bile. Treatments appeared to be unrelated to the development of thyroid cancer since the test materials were dissimilar and the tumor incidence for a given test group was very low.

Two of the spindle cell cancers were found in females, age 13 and 22 months; 4 occurred in males, age 24–34 months. The cancers generally replaced a major portion of the involved thyroid lobe. However, a few normal appearing thyroid follicles were occasionally intermixed with the solid, tumorous portions (Fig. 12). The tumor cells were large, plump and elongated with abundant, pale-pink staining, finely granular cytoplasm (Fig. 13). The nuclei were large and contained irregularly distributed chromatin material. Prominent, pale, acidophilic nuclear inclusions were found in 5 of the tumors (Fig. 14). The thyroid cancer without inclusions was the smallest of the group, occupying approximately $\frac{1}{6}$ of the lobe. Two of the cancers directly invaded adjacent tissues. One of these had metastases to the cervical and anterior mediastinal lymph nodes (Fig. 15). The metastases were structurally similar to the primary tumor, including the presence of follicles filled with colloid. The follicular elements were of the metastasizing struma type seen in human thyroid cancer. As with the papillary and follicular cancers, histologically normal thyroid tissue was present in the non-tumorous portions of the gland (4 animals).

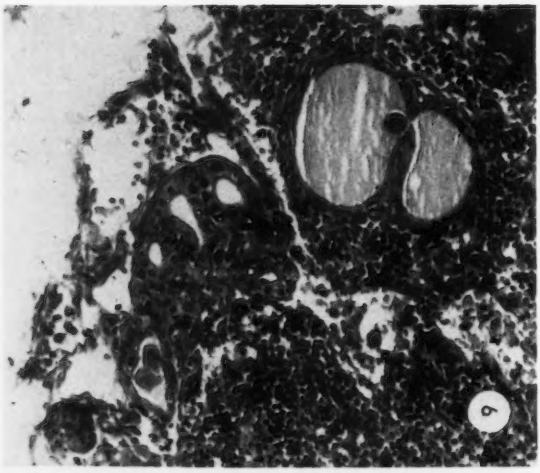
DISCUSSION

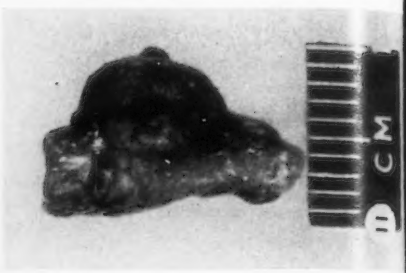
These studies clearly show that the Syrian hamster develops thyroid cancer and thyroid hyperplasia when fed a rice and carrot diet for a prolonged period of time. This phenomenon was observed in 2 experiments utilizing animals from 2 different commercial dealers. The cancers were predominantly well differentiated, follicular adenocarcinomas. A relatively inconspicuous, papillary component was present in most of the primary

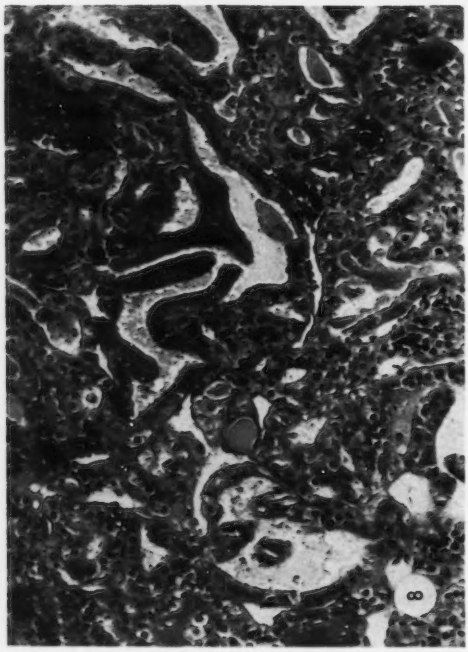
FIG. 5. Representative example of well-differentiated, primary, follicular and papillary adenocarcinoma of the thyroid in a hamster fed the rice and carrot diet for 468 days. The papillary component is more prominent in the illustrated area than in other areas of the gland. The maturity of the tumor is such as to preclude a diagnosis of cancer in the absence of metastases. ×160.

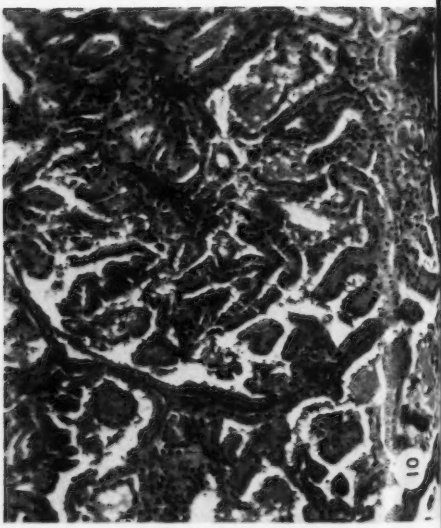
FIG. 6. Area of metastatic, follicular adenocarcinoma in cervical lymph node. The focus is well differentiated and has a benign histologic appearance. Primary tumor is shown in Fig. 5. ×160.

FIG. 7. Histologic appearance of a well-differentiated thyroid cancer. Lymph node metastases were present. ×160.




CM





umors. Metastases were found in the lymph nodes and in the lungs. In nost instances, the cancers were so well differentiated that they could be liagnosed with certainty only when metastases were evident. The metatatic spread of well differentiated tumors also occurs with other hamster ancers. For example, some of the small intestinal adenocarcinomas (9) would have been considered as benign tumors except that metastases to nesenteric lymph nodes were present.

Although the carcinogenic mechanism is unknown, studies in the human nd other animal species suggest that the low iodine content of the diet was an important etiological factor (10, 11). Positive goitrogens and chemical carcinogens were not known to be present. Injected test materials lo not appear to have affected tumor development. The iodine content of the rice and carrot diet is lower than that of diets usually employed in rat experiments (12). Nutritional factors other than iodine are manifestly also deficient in the diet. Further study is necessary to define their role in this neoplastic process as well as to prove the etiological significance of the iodine deficiency.

The response of the hamster to a rice and carrot diet appears to differ markedly from that of the rat. Thyroid enlargement was not noted in any of 120 Sherman rats subjected to essentially the same dietary and other conditions as described for the hamster. Histologic study failed to reveal any thyroid abnormalities in 20 of the rats surviving from 321 to 471 days on the rice and carrot diet. A calcium (13), chloride (14), or other deficiency in the diet may explain why the rats failed to develop thyroidal changes.

No information is available on the serum iodine values of hamsters maintained for a prolonged period on the rice and carrot diet. Short term experiments disclosed that serum total iodine values were low in animals fed this diet for 6 weeks although the protein bound iodine was still unaffected (4).

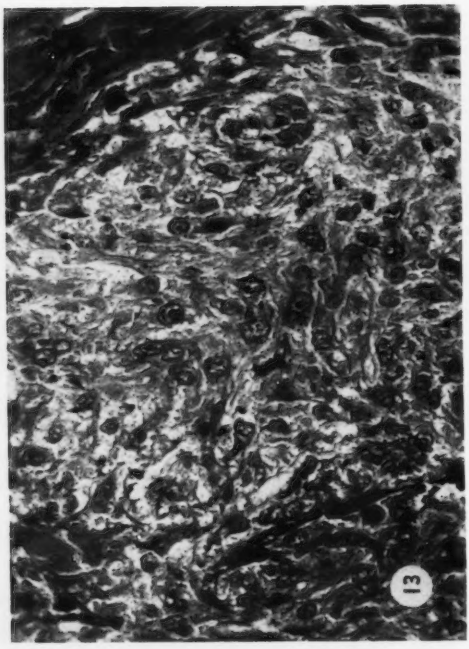
Since spontaneous thyroid cancer was found, it is possible that the

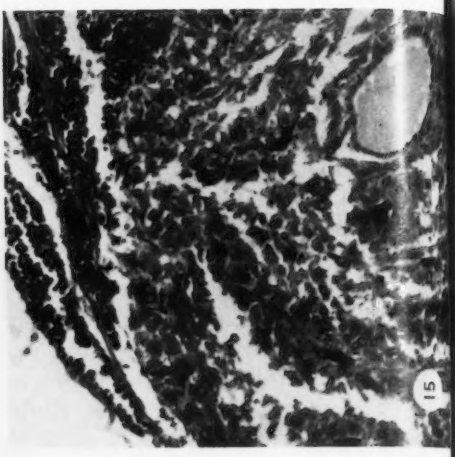
FIG. 8. Less well-differentiated, primary, papillary and follicular adenocarcinoma of the thyroid. ×160.

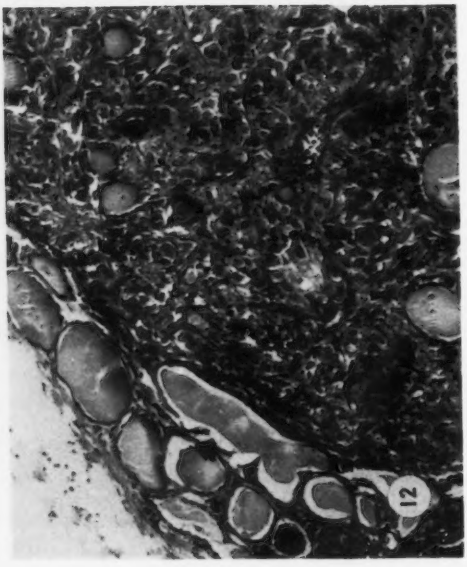
FIG. 9. Well-differentiated, follicular carcinoma metastatic to cervical lymph node. Primary cancer shown in Figure 8. ×160.

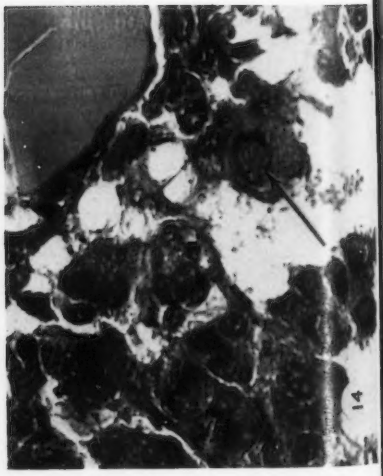
FIG. 10. Histologic appearance of cancer shown in Figure 11. The papillary component is predominant in this area. In other areas, follicular elements were more prominent. ×120.

FIG. 11. Gross appearance of a papillary and follicular adenocarcinoma originating in the left lobe of the thyroid gland. The right lobe is normal both in size and histologic appearance.


13


15


12


14

iodine deficient diet merely increased the incidence of this phenomenon. In this regard, it should be noted that the histologic changes in the thyroids of the induced cancer group are compatible with a TSH effect. The glands were diffusely involved by cancer or cancer associated with hyperplasia of such degree that it could not be differentiated from cancer. In the spontaneous group, many of the cancers were found in an otherwise histologically normal thyroid gland. This suggests the presence of an etiological factor other than TSH stimulation. Histologically, the spontaneous and induced cancers were different. Spindle cell carcinomas were not seen in animals fed the rice and carrot diet. In the spontaneous papillary and follicular carcinomas, the papillary component was more prominent and the cancer less well differentiated than those of the induced group.

Follis (15) has recently confirmed that the Syrian hamster develops hyperplasia of the thyroid gland when fed an iodine deficient diet. He also stressed the similarity of the human and the hamster in the hyperplastic response of their thyroid glands to an iodine deficient diet. Thyroid cancer was not reported to be present in his animals which survived only for as long as 350 days on the diet. Follis found that the pituitary glands of hamsters with thyroid hyperplasia tend to be enlarged and to contain thyrotropic, basophilic cells. Pituitary enlargement was present in many of our animals but study of this problem is yet to be completed.

Acknowledgements

The authors are indebted to Drs. H. T. Randall and I. S. Ravdin for their interest and support; to Dr. William L. Money for many valuable suggestions. Mrs. Alice Gale Mahy gave excellent technical assistance. The bile salts were purified by and obtained from Dr. Ralph Barclay.

REFERENCES

1. Fortner, J. G.: *Cancer* **9**: 1163. 1956.
2. Fortner, J. G.: 3rd National Cancer Conference Proceedings: 807. 1956. J. B. Lippincott Company, Montreal and Philadelphia.
3. Fortner, J. G. and A. C. Allen: *Cancer Research* **18**: 98. 1958.
4. Fortner, J. G., P. A. George and S. S. Sternberg: *Surg. Forum* (1958) **9**: 646. 1959. American College of Surgeons, Chicago, Illinois.

←

Fig. 12. Spindle cell carcinoma of the thyroid gland. Normal appearing thyroid follicles are interspersed with the tumorous portion and are also present peripherally. ×120.

Fig. 13. Histologic features of another spindle cell carcinoma of the thyroid. ×240.

Fig. 14. Intranuclear inclusion present in a large cell (arrow). ×480.

Fig. 15. Cervical lymph node metastases of a spindle cell carcinoma of the thyroid. ×120. A normal appearing thyroid follicle is also present.

5. SUGIURA, S. AND C. P. RHOADS: *Cancer Research* **1**: 3. 1941.
6. BODANSKY, O., R. S. BENUA AND G. PENNACCHIA: *Amer. J. of Clin. Path.* **30**: 375. 1958.
7. FORTNER, J. G.: *Cancer* **10**: 1153. 1957.
8. FORTNER, J. G.: *A.M.A. Arch. Surg.* **77**: 627. 1958.
9. FORTNER, J. G.: *Surgical Forum* (1957) **7**: 193. 1958. American College of Surgeons, Chicago, Illinois.
10. MORRIS, H. P.: *Advances in Cancer Research* **3**: 51. 1955. Academic Press, Inc., New York, New York.
11. AXELROD, A. A. AND C. P. LEBLOND: *Cancer* **8**: 339. 1955.
12. MONEY, W. L., J. E. RALL AND R. W. RAWSON: *J. Clin. Endocrinol. & Metab.* **12**: 1495. 1952.
13. HELLWIG, C. A.: *Am. J. Cancer* **23**: 550. 1935.
14. AXELROD, A. A. AND C. P. LEBLOND: *Canad. M. A. J.* **67**: 675. 1952.
15. FOLLIS, R. H., JR.: *Proc. Soc. Exper. Biol. & Med.* **100**: 203. 1959.

OSTEOPOROSIS IN THE LAYING HEN[1]

MARSHALL R. URIST AND NANCY MARIE DEUTSCH

Division of Orthopedics, Department of Surgery, School of Medicine, University of California, Los Angeles, California

ABSTRACT

Osteoporosis develops in White Leghorn chickens during the first year of egg production and becomes severe during the molt. Immature, and young mature pellets, cockerels, and nonmolting roosters do not have osteoporosis. The disorder appears to be a derangement of the normal process of haversian remodelling: the compact bone is resorbed without osteoclast formation by tunneling operations of blood vessels expanding inside the compact bone; new osteones are not rebuilt to replace old ones. Some of the endocrine organ changes associated with avian osteoporosis are: atrophy of the adrenal cortex and decrease in size and weight of the gonads; reductions in the weight of the thyroids and parathyroids also occur but the decline is relatively slight and inconstant. The level of calcium in the blood plasma is low and either normal or low in ultrafiltrates of serum. Exogenous estrogen (without androgen) increases resorption of cortical bone, accentuates osteoporosis, and may cause fractures from such minor trauma as excitement or handling of the birds.

OSTEOPOROSIS is a disorder of the skeleton, characterized by decrease in the amount of the hard tissue in favor of the soft tissue, without any change in the volume or external configuration of the bones. The White Leghorn hen bred for egg-laying is an animal that spontaneously develops osteoporosis. The metabolic factors responsible for this disorder are obscure. It is well known that the skeleton performs the function of a chemical warehouse for calcium and phosphorus for egg production. In White Leghorns in heavy lay, the rate of reproduction is so great as to impose a definite amount of stress upon the organsim. Added to this, in recent years, culling and breeding have developed the following commercially-advantageous traits: (a) early sexual maturity, (b) constant body weight of approximately 2 Kg., (c) minimum feed consumption, (d) continuous egg production, (e) abstinence from brooding, (f) delayed onset of resting, (g) late molting. It is possible that these traits may lead to further stress, accelerated aging, and susceptibility to disease. Champion birds in competition at State Fairs had a mortality of 10 to 16 per cent before the age of one year (1). Osteoporosis, as will be described in this report, appears before the end of one year in otherwise healthy birds that survive.

Received July 7, 1959.

[1] Supported in part by grants-in-aid from the Josiah Macy, Jr., Foundation of New York, Easter Seal Foundation, Society for Crippled Children and Adults, Ayerst Laboratories, Inc., Arthritis and Rheumatism Foundation, Southern California Chapter, and the Squibb Institute for Medical Research.

It is clear that heavy egg production is associated with hypersecretion of estrogen. This leads to the production of deposits of intramedullary bone, but as will be emphasized in our observations, deleterious changes and osteoporosis develops in cortical bone at the same time. Reports (2, 3) of spontaneous fractures in birds treated with exogenous estrogen to tenderize the meat for marketing, provide evidence of relationship between osteoporosis and hyperestrinism.

There are probably other and as yet unknown factors involved in the pathogenesis of osteoporosis. Meister (4) observed osteoporosis in Canadian Geese and related it to molting. Zahnd (5) described osteoporosis in molting roosters. The occurrence of osteoporosis in healthy molting males reared on adequate diets including optimum vitamin and mineral intake, and no demand for calcium from the skeleton during reproduction, indicates that mineral metabolism is not at fault. The endocrine organs, bone, and blood changes in birds, with and without osteoporosis will be presented below in detail.

METHODS

One hundred White Leghorn birds, 10 immature pullets, 10 roosters, 40 mature pullets, 20 yearling hens, 10 estrogen treated (Estrogenic Substances, Ayerst) yearling hens, and 10 estrogen treated yearling roosters were autopsied and the results of gross examination of the endocrine organs, radiographic examination of the adrenogonadal and skeletal systems, total and ultrafilterable serum calcium, and histological changes are summarized in Table 1. Calcium was measured by the method of Clark and Collip (6). Ultrafiltrates of the serum were obtained by the method of Toribara (7).

RESULTS

Immature Pullets.—The female bird was termed a pullet until she was one year of age. The pullets reached maturity at about five months of age. In the two-week period before they came into lay, the endocrine organs, especially the thyroids, parathyroids, and ovary, increased in size and weight. The size or weight of the adrenal glands was not remarkable. The oviduct was small. The blood calcium was approximately 10.0 mg.% until 7 to 10 days *before* the laying of the first egg. The ultrafilterable calcium was approximately 6.0 mg.% and varied within a small range.

The long bones showed a cortex of solid compact bone, with no secondary haversian systems, and no intramedullary bone (Fig. 1).

Cockerels and Roosters.—The cockerels became sexually mature at five months of age, weighed 1 to 2 Kg. more than pullets and their endocrine organs were proportionally larger in size and heavier in weight than in pullets or hens. The adrenals, however, were 20 to 40% larger per Kg. in mature cockerels than in hens. The range of the weight of the testes was 37 to 42 Gm. The total calcium was 9.5 to 11.0 and the ultrafilterable calcium was 6.0 to 7.2 mg.%.

The cortex of the tibia consisted of solid compact bone with both pri-

TABLE 1

Sex and age	No. birds	Pituitary*	Thyroid*	Parathyroid*	Adrenals*	Ovary (or testes)†	Oviduct†	Total calcium blood‡	UF calcium blood‡	Intramedullary bone§	Osteoporosis§
Immature Pullets	10	5– 7	101–120	12–20	102–125	0.5–0.8	0.4–0.7	9.8– 11.8	5.2–7.4	0.0	0.7–1.0
Roosters	10	9–12	170–288	14–24	200–290	37–42	—	9.5– 11.0	6.8–7.2	0.0	1.0–1.5
Mature Pullets	40	8–10	124–202	15–22	134–298	34–44	31–112	12.7– 35.5	6.3–8.6	0.5–2.0	0.2–1.0 (1+)
Yearling Resting Hens	20	6–10	80–190	6–16	97–172	1–5	2–7	7.6– 11.0	4.8–5.2	0.0–0.5	0.2–0.5 (3+)
Estrogen Treated Hens	10	6–11	81–252	8–20	182–310	1–11	98–189	66.2–120.4	7.3–9.2	2.0–3.5	0.1–0.5 (4+)
Estrogen Treated Roosters	10	8–12	180–301	14–26	210–315	8–15	—	80 –124	7.1–9.4	1.0–1.5	0.5–1.0 (2+)

* Net weight, range, in mg.
† Net weight, range, in gm., including eggs.
‡ Mg.%.
§ Thickness of cortical bone, in mm.

UNIVERSITY OF MICHIGAN

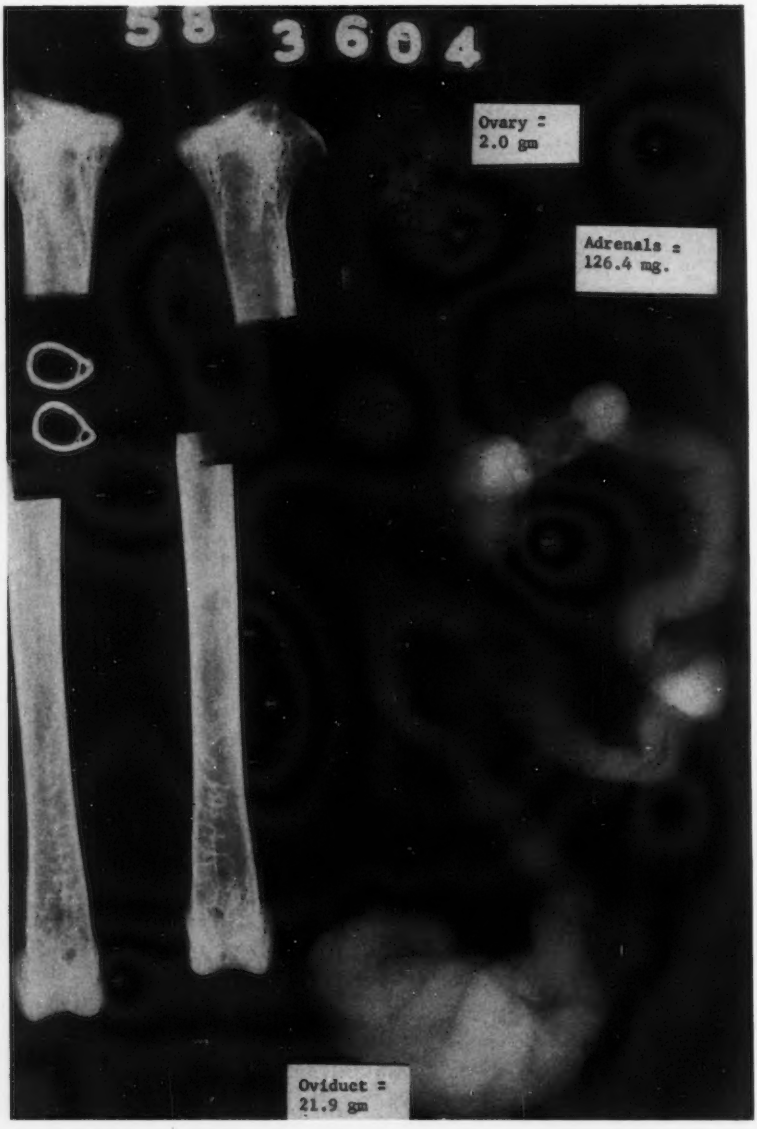


FIG. 1A. Roentgenogram of right and left tibias including cross-sections to show the thickness of the cortex, in a pullet 145 days of age, before the maturation and growth of the first ovum. The adrenals are moderately enlarged and weigh 126.4 mg. The ovary weighs 2.0 gm. or twice as much as it was in a bird 10 days younger without growing comb. The oviduct is greatly enlarged weighing 21.9 gm. and the serum calcium is 17.0 mg.%, both changes resulting from secretion of endogenous estrogen. There is no intramedullary new bone, however, at this early stage of sexual maturation.

mary and secondary haversian systems. At the end of a year when the cockerel molts and becomes a rooster, the cortical bone shows an increase in the large vascular channels but no thinning of the cortex, and thus no appreciable osteoporosis (Fig. 2).

Mature Pullets.—The birds in heavy lay, compared with the immature pullet, the cockerel, and post-molt yearling rooster, showed hypertrophy of all of the endocrine organs. The data in Table 1 show absolute values for a hen weighing approximately 2 Kg. The adrenal glands showed hypertrophy and hyperplasia, not seen in either immature pullets or roosters, or resting hens. The ovaries, including ova storing yellow egg yolk, weighed 34 to 44 gm., but even when the yolks were removed the glands weighed 4 to 6 times more than in resting hens. There was always a hypercalcemia, varying from 12.7 to 35.5 mg.%. The level of the ultrafilterable calcium was slightly elevated in some individuals but rarely more than 1.0 to 2.5 mg.% higher than in immature pullets, roosters, or resting hens.

The cortex of the tibia was only half the thickness of that found in immature birds or adult roosters. In addition, there were enlarged vascular channels and wide tortuous tunnels through the endosteal half of the com-

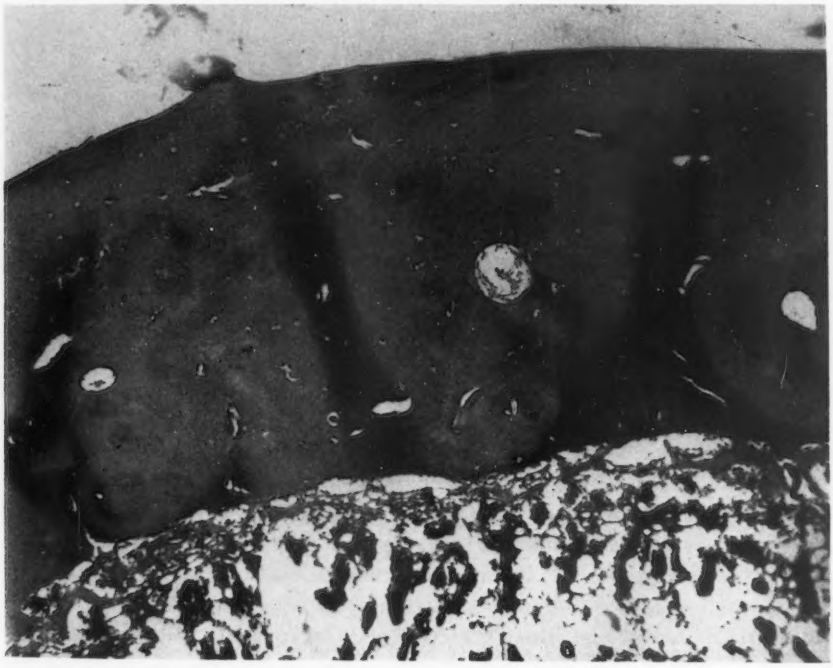


FIG. 1B. Photomicrograph (×70) of the tibia shown in Fig. 1A, showing the dense compact bone of the cortex. There is no evidence of osteoporosis in the pullet before she comes into lay.

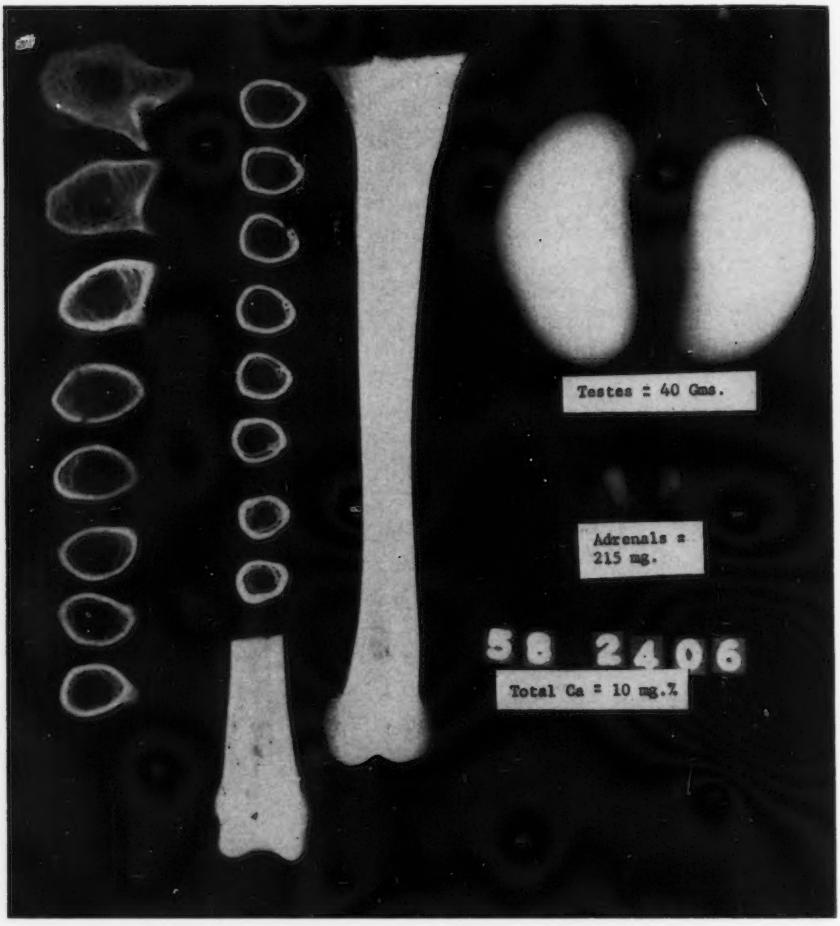


FIG. 2A. Roentgenogram showing cross-sections of the right tibia, the intact left tibia of a rooster, to show the thickness of the cortex. The testes and the adrenal glands are normal size for an adult male.

pacta. There were always deposits of intramedullary bone lining the marrow cavity and inside the enlarged haversian canals (Fig. 3).

Yearling Resting and Molting Hens.—The average laying bird underwent a complete molt at the conclusion of the first year of laying. During the hot weather of the late summer and early fall in our laboratory in Southern California, some of the birds ceased to lay for periods from one to two months. The weight of the endocrine organs of these birds was interesting in that the thyroid and adrenal glands were small or atrophied; the parathyroids were half the size of those in laying hens. The ovaries weighed as

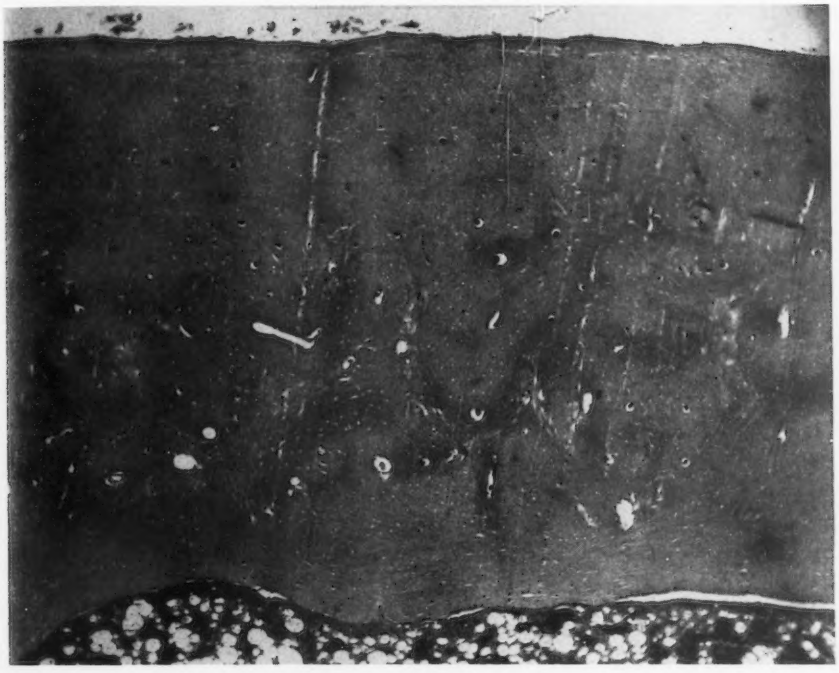


FIG. 2B. Photomicrograph (×70) of the cortex in cross-section shown in Fig. 2A. There is solid compact bone and no osteoporosis in the mature young rooster.

little as 1.0 gm. and the oviducts as small as 2.0 gm., indicating a low level of estrogen secretion. The total calcium was also low, varying from 7.6 to 11.0 mg.%; the level of the ultrafilterable calcium was 4.8 to 5.2 and reduced in proportion to the level of the total calcium.

The tibias were extremely osteoporotic. The bone tissue was fully calcified but so light and brittle, that it broke into fragments when cut with a knife. There were many enlarged vascular channels. None, however, contained deposits of intramedullary bone and few showed osteones with areas of active new bone formation. There was no apparent increase in the number of osteoclasts. The bone matrix was fully calcified. The microscopic picture in resting (menopausal) or molting birds, with severe osteoporosis compared with laying pullets with mild osteoporosis suggests that the rebuilding phase of haversian remodelling failed to materialize and refill old vascular channels with new bone, or replace bone previously lost by resorption (Fig. 4).

Estrogen-Treated Hens.—An intramuscular injection of Estrogenic Substances (Ayerst), 100 mg. per week, for 4 weeks, produced specific defects in the structure of the cortical bone. Estrogen treatment of yearlings (pullets

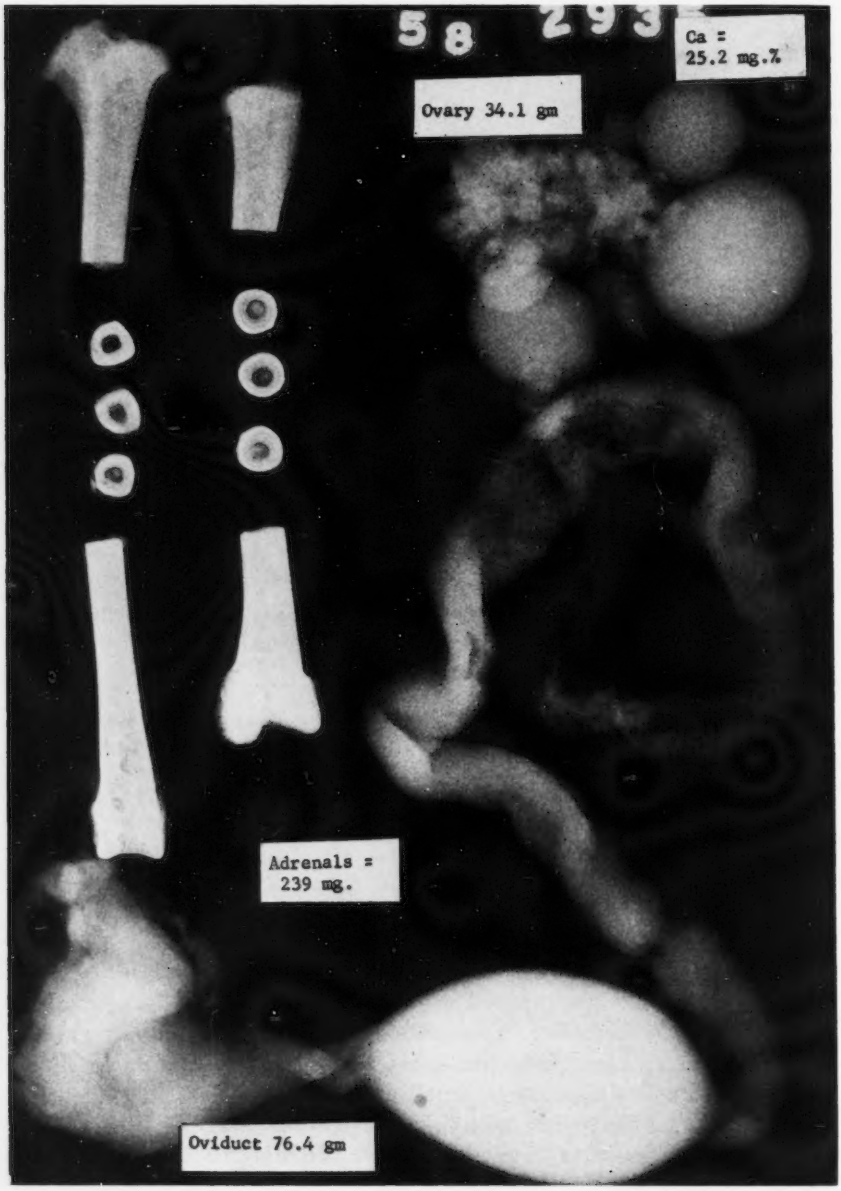


FIG. 3A. Roentgenogram showing cross-sections of the tibia and femur (upper left) of a mature hen, 11 months of age, in heavy lay showing a layer of spongy bone on the endosteal surface of the cortex. The ovaries show ova in all stages of growth. The isthmus of the oviduct contains an egg with a complete covering of albumin. The adrenals are greatly hypertrophied, weighing 239 mg. or more than 100% larger than in immature pullets.

FIG. 3B. Photomicrograph (×70) of the cross-section of the tibia shown in Fig. 3A. The cortex is one half as thick as in the rooster and contains many osteones with enlarged vascular channels and a thin layer of intramedullary bone. The marrow cavity also contains a thick layer of intramedullary bone.

are less affected and can continue to lay) caused the ovaries to become reduced in size (possibly due to suppression of the pituitary), while the oviduct increased in size and weight. The adrenal glands (and the thyroid in some instances) also increased in size and weight. There was no apparent effect on the pituitary or parathyroids. There was a pronounced hypercalcemia, varying from 66.2 to 120.4 mg.%; the ultrafilterable calcium was only 1.0 to 2.0 mg.% higher than in non-estrogen treated birds.

The cortex of the tibia and femur showed marked reduction in thickness and large defects filled with intramedullary bone. The marrow cavity was completely occluded with intramedullary new bone. The combination of compact bone and spongy intramedullary bone was weaker, rather than stronger, than the solid compact bone alone. The long bones were as brittle as osteoporotic bone and easily broken following treatment with large doses of exogenous estrogen (Fig. 5).

Estrogen-Treated Rooster.—Intramuscular injection of 100 mg. of Estrogenic Substances per week in roosters produced all the changes that were observed in hens. The testes were precipitously reduced in size from approximately 40 gm. to 10 gm. within two weeks. The adrenal glands be-

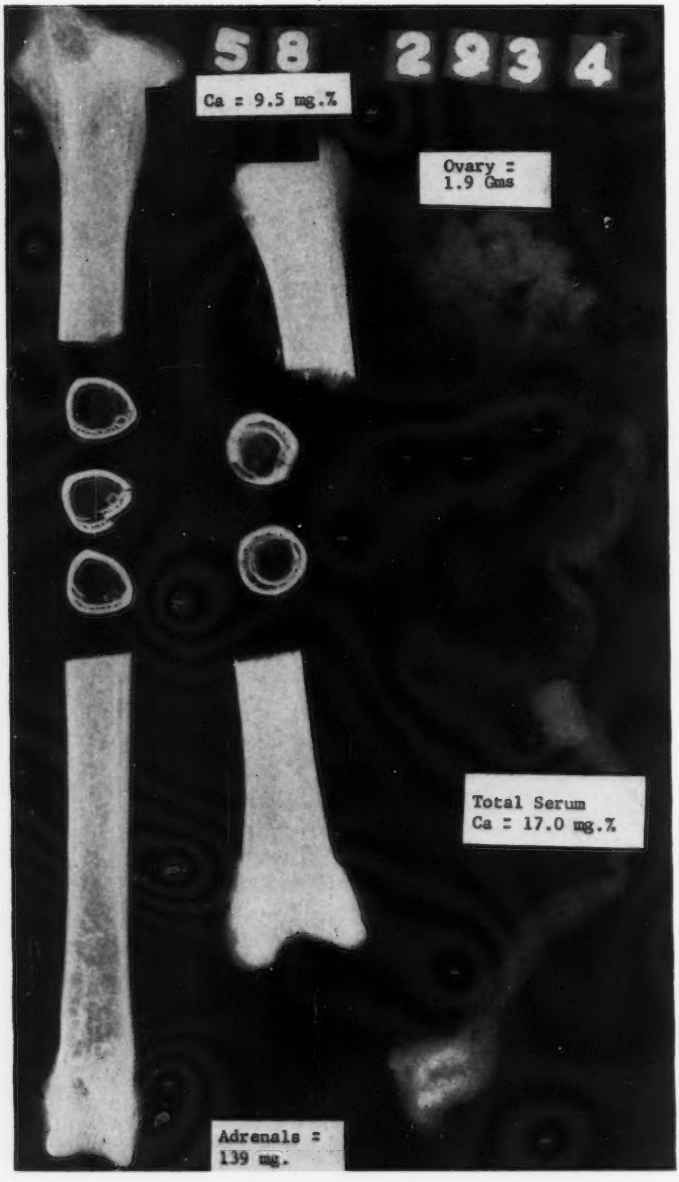


FIG. 4A. Roentgenogram showing a cross-section of the tibia and femur of hen after the first year of heavy egg production. The cortex is thin and brittle. There is longitudinal splitting and enlarged vascular channels in the compact bone. There is no intramedullary bone remaining inside the marrow cavity. The ovaries are inactive and contain no growing ova. The oviduct is atrophied and the serum calcium was 9.5 mg.% indicating a lower level of secretion of estrogen. The adrenal glands are relatively small in size and light in weight.

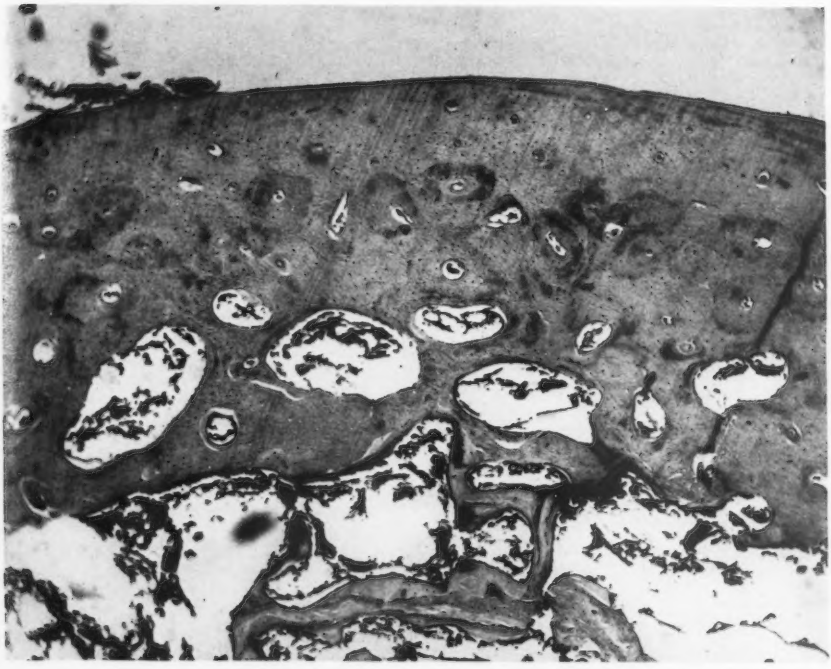


FIG. 4B. Photomicrograph (×70) showing the cortex less than half that of normal. The haversian canals are enlarged and filled with blood vessels, bone marrow, but no osteoclasts. These channels, normally filled with the intramedullary bone deposits of the laying hen, remain to be refilled with new compact bone; thus the rebuilding phase of the process of haversian remodelling is absent. The persistence of these channels on the endosteal side without any loss of osseous tissue on the periosteal side of the bone, is pathognomonic of osteoporosis.

came hyperplastic and hypertrophied. The changes in the serum have already been described in detail in a previous paper (6).

The cortex of the tibia showed resorption of compact bone and enlarged vascular channels within three weeks. After three weeks, a thin layer of intramedullary bone formed as a lining of the marrow cavity. Prolonged treatment with estrogen increased the amount of erosion of the cortex and replacement of compact bone with spongy bone. When estrogen treatment was discontinued, the intramedullary bone was resorbed and the remaining cortical bone was porous, brittle, and slow to repair.

DISCUSSION

One of the outstanding features of avian osteoporosis, as in man and other animals, is the decrease in the thickness of the cortex of the bones. The compact bone is also more porous. The cortical bone is resorbed from

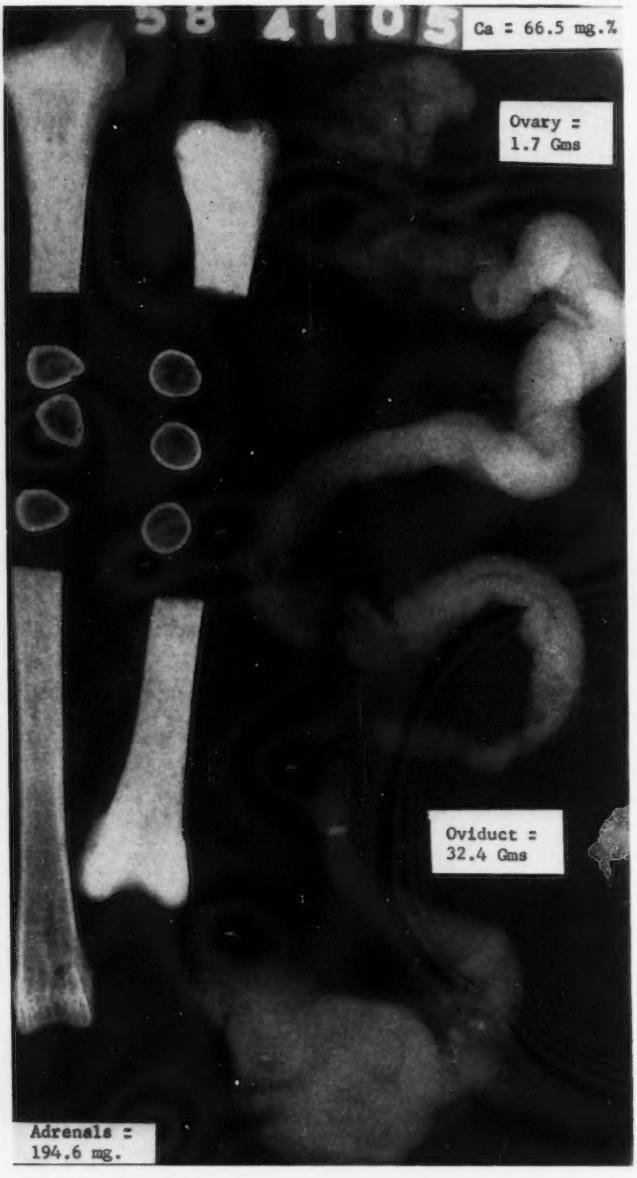


FIG. 5A. Roentgenogram showing cross-sections of the tibia and femur of yearling hen 6 days after an injection of 125 mg. of Estrogenic Substances (Ayerst). Note further decrease in thickness of the cortex while the marrow cavity completely filled with intramedullary new bone. The ovary is atrophied and weighs only 1.7 gm. The oviduct is large and weighs 32.4 grams without any egg in it. The adrenals are moderately enlarged and weigh 194.6 mg.

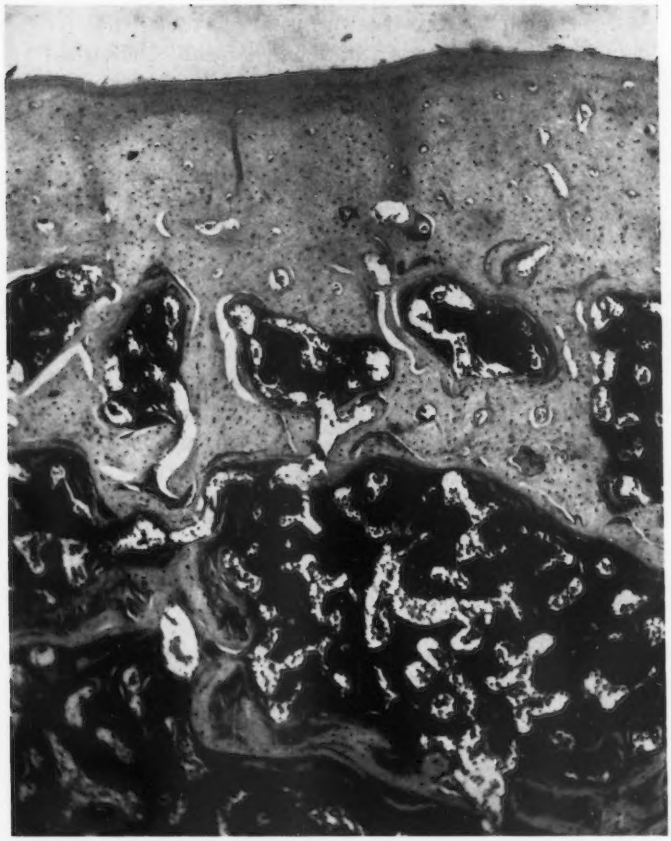


FIG. 5B. Photomicrograph (×70) of the specimen shown in Fig. 5A. The thin brittle cortex contains channels filled with intramedullary new bone. The marrow cavity is also occupied by deposits of intramedullary bone, deep-blue and metachromatic staining with azure. The bone produced under the influence of estrogen is soft, has no mechanical function, and serves chiefly as a reservoir for calcium to be transmitted from the hen to egg shell. In this condition, although the cortex is heavily calcified, the compact bone is osteoporotic and brittle and susceptible to spontaneous fractures.

within, enlarging the diameter of the marrow cavity. The bone tissue that is present is fully calcified, possibly even hypercalcified so that the per cent ash may be unchanged or slightly increased; for this reason, in the past, determinations of the bone ash have not disclosed the presence of osteoporosis and fail to explain the increased fragility of the bones in osteoporosis.

The cause of spontaneous fractures in osteoporosis can readily be seen in cross-sections of the cortical bone with the aid of correlated radiographic and histologic methods. The cortex appears to be resorbed from within in a

centrifugal direction until the structure is so weakened that it is unable to withstand such slight amount of trauma as handling the birds to put them in crates for the market. The cortical bone is resorbed and the haversian canals are enlarged by some unknown mechanism that does not require the action of osteoclasts. The matrix and the mineral are removed together; *halisteresis*, the term literally meaning salt deprivation, may apply to the bone changes in osteomalacia, but not osteoporosis in which the bones are fully calcified, hard, and brittle.

Avian osteoporosis has been produced by transferring hens to low calcium diets (8). This condition was accompanied by cessation of egg production. The adrenal glands, however, were either normal or large in size. The birds showed first an increase and then a decrease in the volume of intramedullary bone, suggesting a secondary effect upon the level of F.S.H. or estrogen production by the ovaries. The parathyroids were generally increased in size and weight. These observations suggest that low calcium intake is associated with multiple and diverse endocrine reactions; in itself, a calcium-deficient diet is probably not sufficient to produce avian osteoporosis.

Resting or molting birds with severe osteoporosis showed either prelaying or low levels of the serum calcium. Egg production or estrogen treatment, as described in previous reports (9), increased the level of the total calcium owing to the production of a phosphoprotein X_1, associated with a phospholipid-lipoglyco-protein, X_2, produced by the liver. The calcium that was added to the serum was absorbed from the gut and from cancellous bone but was very likely also mobilized from cortical bone and contributed to the production of osteoporosis.

Egg production and estrogen treatment were also associated with formation of a system of intramedullary new bone. This deposit, similar to a female secondary sex characteristic, rises and falls in volume with the level of production of estrogen. The function is to store calcium for calcification of egg shells. The cortex shows osteoporosis at the same time that the intramedullary deposits grow in volume, and in fact displace compact bone. Hence, it is likely that the animal transfers calcium from the compact bone to the intramedullary bone.

Osteoporosis has been attributed to many causes. One mechanism, common to all causes, has been suggested in the recent literature; this is the unopposed action of 17-hydroxycorticosterone and other glucocorticoids produced by the adrenal cortex (10). In avian species, corticosterone is produced in large amounts during reproduction. According to Reifenstein (11) sex hormones, estrogens and especially androgens, oppose the anti-anabolic effect of glucocorticoid hormones upon bone tissue. The prototype of this form of osteoporosis and the evidence for the preventative action of sex hormones, may be seen in experimental and clinical Cushing's Syndrome.

Osteoporosis in White Leghorn hens differs from osteoporosis in Cushing's Syndrome in man in that it is associated with small rather than large adrenal glands. On one hand, during the period of reproduction, when estrogen is secreted in large amounts, the adrenals are hypertrophied. On the other hand, when reproduction ceases, and molting is at its height, the adrenals are atrophied. Avian osteoporosis is more like senile osteoporosis (12) than hyperadrenalcortical osteoporosis in that it is found when the adrenals are atrophied rather than hypertrophied. The intermediate steps and the metabolic interrelations leading to osteoporosis are not known. The amount of adrenal glucocorticoid hormones in plasma and excreta of laying hens and estrogen treated birds is now being measured in our laboratory. The plasma levels, thus far, do not appear to be abnormal. The excreta present technical difficulties we have not yet been able to solve. Experiments with ACTH and exogenous adrenal steroids in birds with and without osteoporosis will be presented in later communications (13).

REFERENCES

1. Jull, M. A.: Successful Poultry Management. Second Edition. New York, McGraw-Hill Book Co., 1951.
2. Fry, J. L. and W. J. Stadelman: *Poult. Sci.* **37:** 67. 1958.
3. Gassner, F. X., E. C. Reifenstein, J. W. Algeo and W. E. Mattox: *Rec. Prog. in Hormone Res.* **14:** 183. 1958.
4. Meister, W.: *Anat. Rec.* **111:** 1. 1951.
5. Zahnd, J. P.: *C. Rend. Soc. Biol.* **148:** 1491. 1954.
6. Clark, E. P. and J. B. Collip: *J. Biol. Chem.* **63:** 461. 1925.
7. Toribara, T. Y.: *Anal. Chem.* **25:** 1286. 1953.
8. Urist, M. R.: *Rec. Prog. in Hormone Res.* **15:** 455. 1959.
9. Urist, M. R., O. A. Schjeide and F. C. McLean: *Endocrinology* **63:** 570. 1958.
10. Urist, M. R.: *J.A.M.A.* **169:** 710. 1959.
11. Reifenstein, E. C., Jr.: *Clin. Orthoped.* **10:** 206. 1957.
12. Urist, M. R.: Lankenau Hospital Conference on Bone as a Tissue. Symposium on Osteoporosis. Phila., Pa., Blakiston Co., 1959.
13. Urist, M. R. and N. M. Deutsch: In Press.

A STUDY OF THE SURVIVAL AND FUNCTION OF HOMOGRAFTED THYROID TISSUE IN MEMBRANE FILTER CHAMBERS IN THE RAT[1]

JOHN R. BROOKS, GEORGE J. HILL, II, ALBERT DE'SCOVILLE, JULIO PRIARIO, DIANE CROCKER AND HERBERT A. SELENKOW

Department of Surgery and Medicine, Harvard Medical School and Departments of Surgery and Medicine, Peter Bent Brigham Hospital, Boston, Massachusetts

ABSTRACT

Thyroid tissue has been successfully homografted in Millipore diffusion chambers between rats that have been shown routinely to reject skin homografts. In such grafted animals, histological sections of chambers removed have revealed normal thyroid tissue and colloid-filled acini in 70% at 1 month and 44% at 4 months. Further corroborative evidence of functional viability has been based on a significant uptake of a tracer dose of iodine^{131} given prior to removal of the chambers, and on radio-autographs obtained.

Problems relating to membrane thickness and to chamber breakage are discussed and the question of possible tissue adaptation to the host with the passage of time is raised.

SUCCESSFUL experiments in the transplantation of tissues within Millipore filter chambers have been reported by a number of investigators in recent years. The initial observations by Algire *et al.* (1–3) demonstrated that these chambers could be used to protect a variety of normal and neoplastic tissues in homo- and heterotransplantation. Others (4, 5) studied the behavior of tissues which had been placed within these chambers. These chambers provide a useful means for distinguishing between cellular and noncellular aspects of the forces which act upon tissues (6). When they are constructed from membrane filters with the proper pore-size, the chambers will admit particles of molecular size while restricting the passage of cells.

Attempts have been made in a number of laboratories to use the Millipore chamber technique in the transplantation of endocrine tissue. A variable degree of success has greeted these efforts. Sturgis and Castellanos have successfully performed ovarian homotransplantation in the rat (7), and ovarian heterotransplantation from a human to a monkey (8). However, human ovarian homotransplantation has thus far been of limited success (8). Jordan (9) was unable to secure any success in the autotrans-

Received July 16, 1959.

[1] Supported in part by Grants A-1425 and HF-8950 from the United States Public Health Service, and supported in part by a grant from the Atomic Energy Commission

plantation of parathyroid tissue within diffusion chambers, and the preliminary observations of Stone and Eyerling (10) have not been confirmed by further reports. Hallin and Swan (11) reported a variable degree of success in their attempts to perform homotransplantation of ovary, testis, prostate, thyroid, parathyroid and pancreatic islet cells in Millipore chambers in the dog. Diffusion chamber techniques have been utilized in the isotransplantation (12) and homotransplantation (13) of thyroid by other workers, and preliminary observations of adrenal homotransplantation have been reported (13).

In this report additional observations are presented in the studies of the homotransplantation of thyroid tissue in the albino rat. The purposes of this experiment are: 1) to compare the survival of the transplanted tissue at monthly intervals, and 2) to evaluate the functional status of the transplanted tissue.

MATERIALS AND METHODS

The donor and recipient animals in this experiment were adult female albino rats of the Sprague-Dawley strain.[2] The recipients were a group of 67 rats which had been thyroidectomized 3–8 months prior to receiving the transplanted thyroid tissues. Each recipient animal had received an intraperitoneal injection of 200 μc of I^{131} prior to receiving the transplanted thyroid, to decrease the amount of residual endogenous thyroid tissue. The large dose of I^{131} was given 2–5 months prior to the grafting procedure. The rats which served as donors were obtained from the same breeding farm as the recipients. The donors were younger animals (2–3 months old) and both donors and recipients were the products of purely random breeding in a large colony.

The Millipore filter chambers were constructed using the technique of Algire (1). The chambers utilized HA Millipore (14), which has an effective pore-size of approximately 0.45μ and is $150 \pm 10\mu$ thick. Figure 1 illustrates the method of assembly of the Lucite rings and Millipore circles into the shallow receptacles which comprise the chamber. The chambers were sealed with 2% lucite in acetone after the tissue was inserted. The empty chambers were sterilized by exposure to the vapors of ethylene oxide for at least 12 hours.

The operations upon the rats were carried out with clean instruments and clean technique, though no attempts were made to maintain the sterility of anything except the chambers. The donor and recipient rats were simultaneously anesthetized with open drop ether. Then, using iridectomy scissors and exceedingly fine toothed forceps, each thyroid lobe of the donor was removed and minced into fragments 0.5–1.0 mm. in diameter. The tissue was repeatedly moistened with Hank's balanced salt solution and the minced tissue was suspended in a drop of this solution before it was sealed in the chamber. All of the tissue from an individual thyroid lobe was placed into a single chamber, and the 2 chambers were then inserted into the subcutaneous tissues of the lateral abdominal wall. An indirect route was used in order to avoid premature extrusion of the chambers. A ventral midline incision was made and the abdominal contents were retracted to the side; using this exposure, a second incision was made through the flank musculature, so that a pocket could be opened up in the subcutaneous space. This procedure was repeated to produce a second pocket in the opposite flank. After the cham-

[2] Obtained from the Charles River Laboratories, 1093 Beacon Street, Boston, Massachusetts.

bers had been inserted in the subcutaneous spaces, all three incisions were closed with 0000 cotton.

The recipients were divided into four groups of approximately equal size, and the groups were sacrificed at monthly intervals. Twenty-four hours before the animals were sacrificed, they were injected intraperitoneally with 3μc of I^{131} in 1.0 ml. of water. The chambers were divided transversely after their removal; one half was placed in formalin for later histologic examination, and the other half was digested in 10% NaOH (100° C)

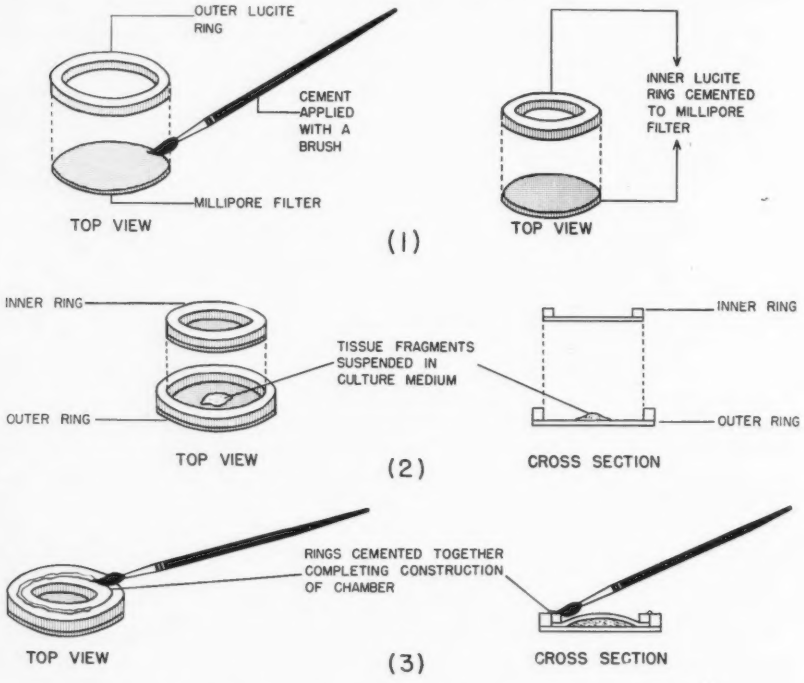


FIG. 1. Steps in construction of the membrane filter chambers used in tissue homotransplantation.

and counted in a well scintillator. Microautoradiographs were prepared with tissue from various rats chosen at random, to study the areas of localization of radioactivity within the chamber and in the peritracheal areas.

In preparing the microautoradiographs, the tissue was fixed in formalin overnight, and the next day it was carried through the usual paraffin imbedding process and sectioned into slices 4 μ thick. These slices were placed in a light-proof box against Kodak Type A 1×3 inch autographic plates for 8 days. The plates were then developed with Kodak D-19 developer and acid fixer.

Controls were established by a number of concurrent experiments, which may be summarized as follows: 1) Attempts to perform skin homografts were made in 92 adult Sprague-Dawley rats. No permanent survival of homotransplanted skin was obtained, although a technique of skin autotransplantation was developed which gave 98% successful transplantation. 2) Previous efforts to perform homotransplantation of the rat adrenal gland without the protection of Millipore chambers had been a complete failure.

histologically and functionally, in 41 rats. 3) The average weight of transplated thyroid tissue was 17.2±2.0 mg. per rat. A portion of skeletal muscle of similar size was taken from each recipient rat to serve as a control for the I^{131} uptake studies. The average weight of 5 of these muscle fragments was found to be 17.4 mg.

RESULTS

Fifty-four rats received homotransplanted thyroid tissue within Millipore chambers. Ten rats died early in the experiment and were not included in the data. The remaining 44 rats received a total of 87 Millipore chambers

TABLE 1. SUMMARY OF OBSERVATIONS UPON 84 MILLIPORE CHAMBERS CONTAINING HOMOGRAFTED RAT THYROID TISSUE. RATS WERE SACRIFICED AT MONTHLY INTERVALS FOR HISTOLOGIC EXAMINATION OF THE TRANSPLANTED TISSUE

	Removal time following transplantation (months)				
	1	2	3	4	Total
Number of chambers examined each month	22	23	23	16	84
Containing viable thyroid	14	16	10	7	47
Containing leukocytes, fibrous or necrotic tissue	8	7	13	9	37
Per cent of chambers which contain viable thyroid	64	70	44	44	56

containing thyroid tissue and were sacrificed at set intervals. Eighty-four of these chambers were examined histologically. Three chambers were damaged in the process of histological preparation. The observations upon 84 chambers are summarized in Table 1.

The microscopic observations are based upon block sections taken at 2–4 points in the half-chamber which had been placed in formalin. Representative illustrations of thyroid and fibrous tissue elements in the chambers are shown in Figures 2–4. Another interesting finding was the presence of giant follicles within 6 chambers. Often these follicles showed squamous metaplasia, and usually they did not contain colloid (Fig. 5).

The 24 hour uptake of I^{131} was measured in 77 half-chambers. Of these, 43% showed an uptake at least 4 times greater than the specimens of skeletal muscle from the same animal. The average weight of these muscle samples was 17.4 mg. The average total weight of original thyroid tissue transplanted in 2 chambers was 17.2 mg. (see above). At the completion of each experiment, one-half of each chamber was counted for radio-activity. Therefore, at best, approximately 4 mg. of original tissue was being counted. This fails to take into consideration the consistent and considerable loss of tissue weight that occurrs within the chamber with the passage of time subsequent to grafting. For technical reasons, the tissue recovered following chamber removal could not be separated from its millipore membrane and weighed. However, it represented only a fraction of that originally transplanted. Millipore chambers left *in situ* without thyroid tissue failed to concentrate the radioactive iodine.

It was interesting to discover that histologic evidence of viable thyroid

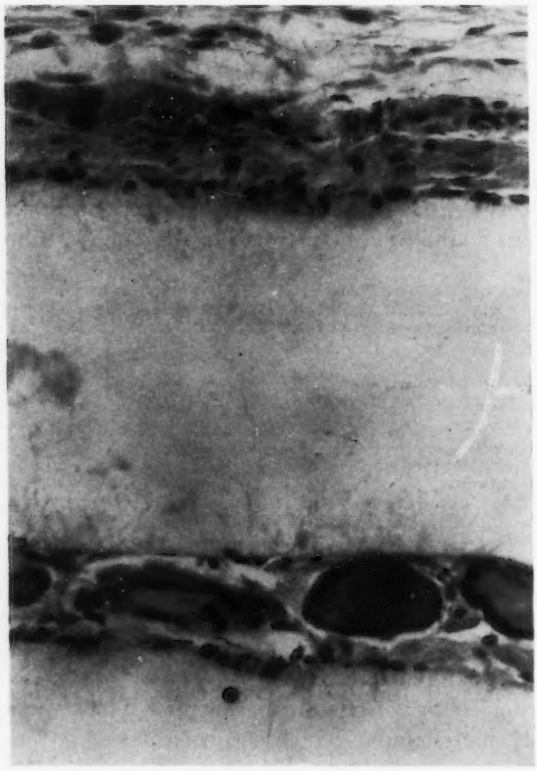


FIG. 2. Thyroid homograft removed one month after implantation, showing the Millipore chamber and surrounding tissues of the recipient animal in cross-section. Note the fibrous tissue of the host at the top, the 150 μ thick HA Millipore, and the layer of transplanted tissue showing thyroid follicles. (90×)

tissue could be demonstrated in only two-thirds of the chambers which concentrated I^{131}. However, the 7 chambers which contained luxuriant growth of thyroid tissue and also demonstrated the greatest degree of I^{131} uptake had radio-active iodine uptakes >0.4% in 24 hours. Three of these chambers were studied with microautoradiographs, and the radioactivity was demonstrated to be localized in the area of the follicles in these chambers (Fig. 6). In contrast, microautoradiographs of chambers which contained only small amounts of thyroid tissue or which were frankly rejected invariably failed to show localization of radio-activity within the chambers.

An unexpected result of the experiment was the demonstration that a certain percentage of the chambers which contained viable thyroid tissue were either broken open or contained capillaries with erythrocytes (Fig. 7). Chambers were classified as "broken" if they exhibited any of three features: 1) microscopic discontinuity of the membrane when it was removed

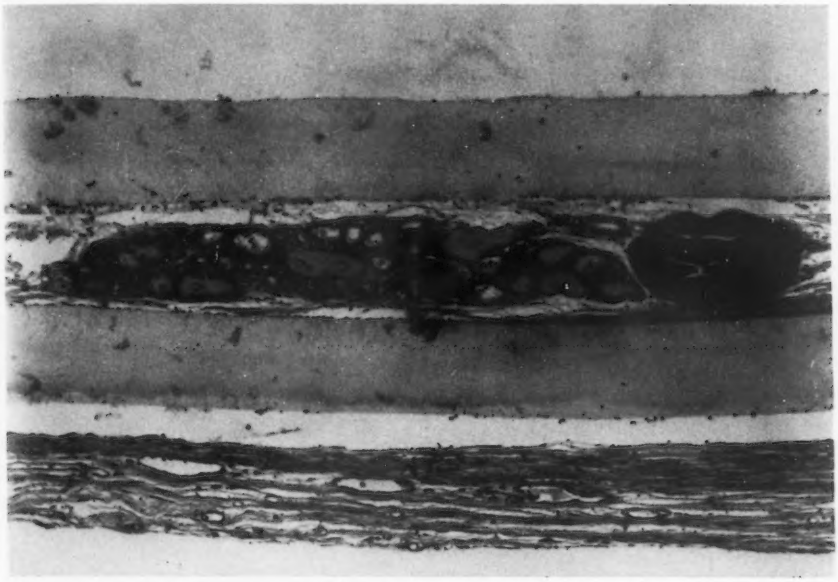


FIG. 3. Thyroid homograft removed four months after implantation, showing considerable amounts of thyroid tissue between the two surfaces of the diffusion chamber, which is seen here in cross-section. (37×.)

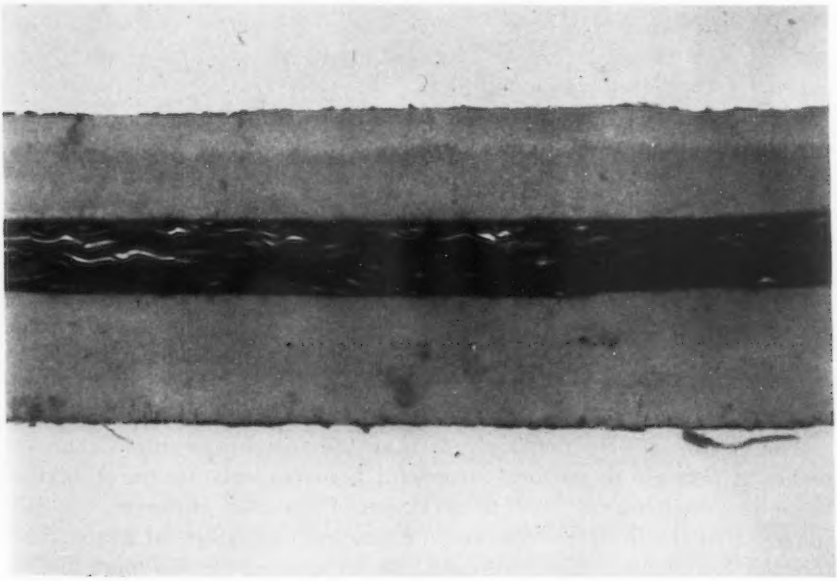


FIG. 4. Thyroid homograft removed two months after implantation, with only a thin layer of fibrous tissue in the diffusion chamber.

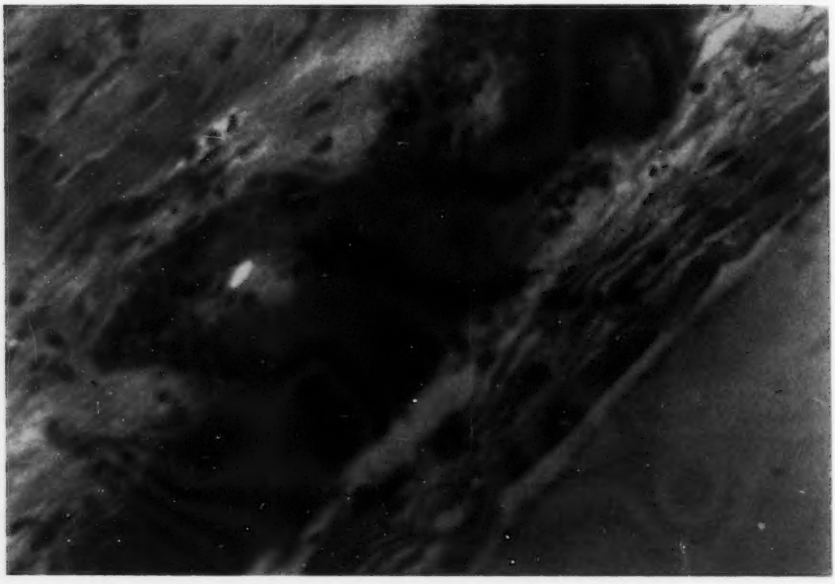


FIG. 5. Squamous metaplasia, occurring in a gaint thyroid follicle, in homografted tissue within a Millipore chamber, removed three months after implantation. This chamber was also found to have been broken open (Fig. 8). (97×.)

from the experimental animal; 2) microscopic evidence of a well-established break in the membrane which admitted fibrous tissue (Fig. 8), or 3) polymorphonuclear leukocytes within the chamber. Thirty-seven per cent of the chambers were thus classified as "broken". However, two-thirds of these chambers contained thyroid tissue which was normal in appearance. Sixteen per cent (13/84) of the chambers contained capillaries with erythrocytes, and 12 of these chambers contained viable thyroid tissue.

The 10 rats which died before their chambers were removed were excluded from the experiment, because of the difficulty in obtaining prompt postmortem examinations. The causes of death of these animals were pneumonia, in four; unknown cause, in three; brain abscess, in one; strangulated incisional hernia, in one; and peritonitis, in one.

DISCUSSION

The results clearly demonstrate that the membrane filter technique makes it possible to perform successful homotransplantation of thyroid tissue in the albino rat, with preservation of function. However, the data suggest that the initial success may be tempered by failure at a later date through one or more mechanisms: 1) In some cases the Millipore filter is apparently altered, becomes permeable to cellular material and gradually becomes basophilic, cracked and wrinkled or broken. 2) In most chambers

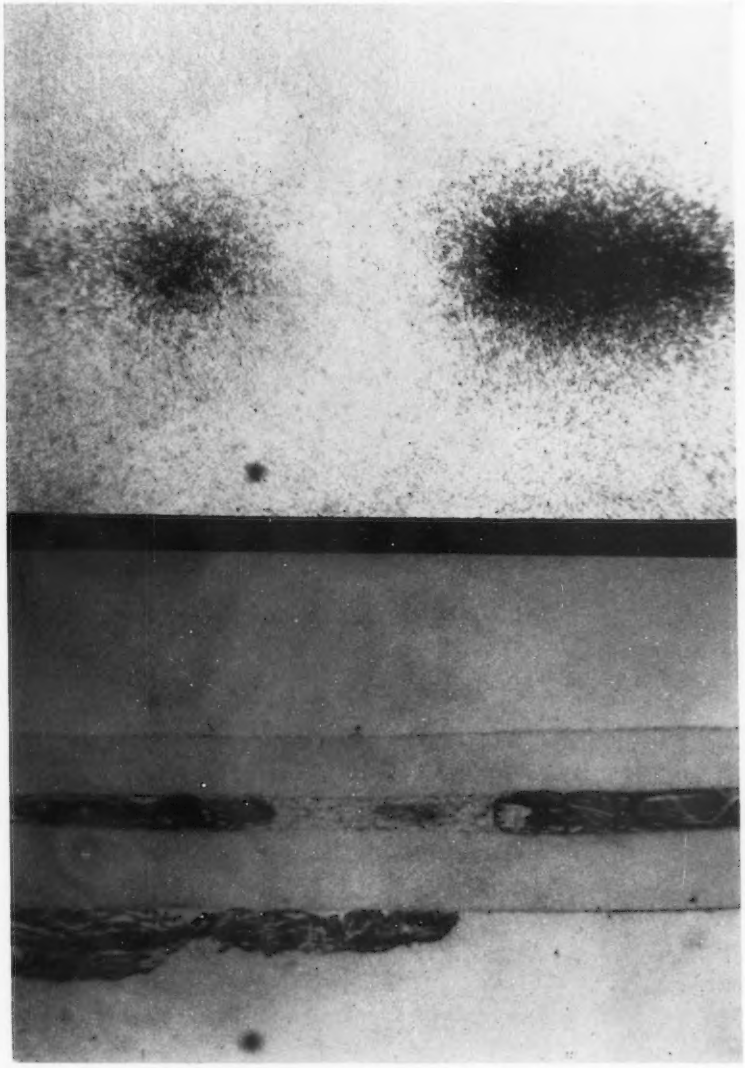


FIG. 6. Microautoradiograph and corresponding tissue section, showing localization of radioactivity in follicular areas of the homotransplanted rat tissue. Homotransplant, within HA filter chamber, was removed two months after implantation. (36×.)

removed after 3–4 months in the host, the amount of thyroid tissue has decreased and there is a progressive increase in the amount of fibrous stroma. The increased proportion of fibrous tissue is apparently the most serious aspect of the problem, for the number of failures due to broken chambers

FIG. 7. Diffusion chamber containing homografted thyroid tissue, removed one month after implantation, showing erythrocytes in capillaries surrounding histologically identifiable thyroid tissue. (77×.)

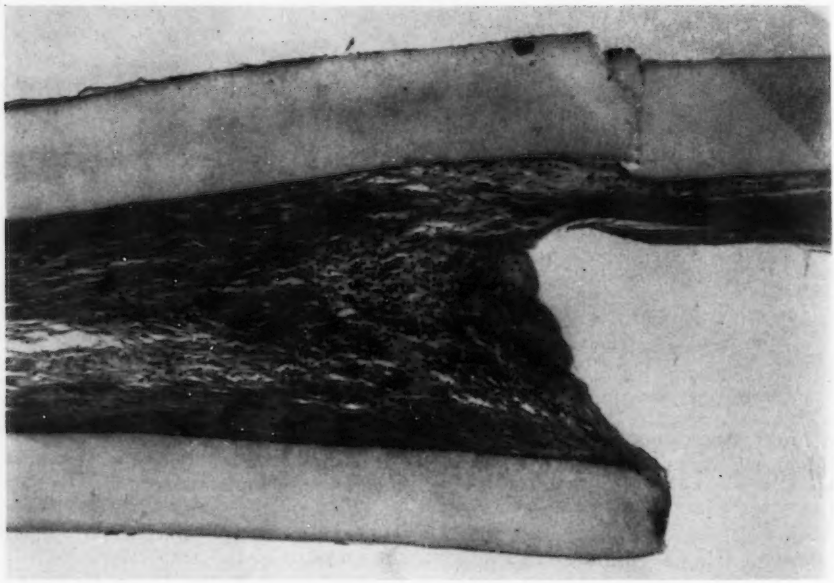


FIG. 8. Diffusion chamber, showing broken membrane filter, admitting fibrous tissue and lymphocytes. The chamber, containing homografted rat thyroid, had been implanted three months previously. (37×.)

does not appear to increase significantly from month to month. In addition, it is possible to strengthen the membrane by incorporating in it a monofilament nylon mesh. In contrast, no adequate method has been worked out to prevent the gradual increase in fibrous tissue in the chambers. The fibrous tissue may well be derived from thyroid stroma. This would represent a situation analogous to the well-documented modulation of tissues cultured *in vitro* under a variety of conditions (15).

The progressive increase in the fibrous elements in the chamber might represent a delayed response to the initial trauma which the tissue undergoes in the operation of removing and preparing the explant. Another part of the initial trauma is the period of adjustment to a delayed diffusion of oxygen to the devascularized tissue within the Millipore chamber. Later in the course of the life of the transplanted tissue, it is conceivable that the tissue may be subjected to chronic hypoxia because of the membrane filter and scar tissues surrounding it. This chronic hypoxia might cause death and fibrosis of the tissue.

The existence of capillaries containing erythrocytes in the Millipores (Fig. 7) has been a unique finding in this experiment. It is unlikely that the capillaries and cells are developed from vascular elements in the donor tissue, for in no cases have primitive erythrocytes or leukocytes been seen within the chambers. Therefore, the capillaries appear to be *prima facie* evidence that the chamber has been broken open, and that host tissue is in contact with the grafted tissue. The fact that there is usually no inflammatory response in such cases, either granulocytic or lymphocytic, suggests that the host is tolerant of the grafted tissue. It is conceivable that the transplantation with the Millipore chamber acted in some manner as a conditioning influence. An analogous situation is the "adaptation" of transplanted tumors in rodents, as demonstrated by Koprowski (16). In his experiments, the tumors were partially protected by intracerebral passage for several generations, and they were finally "adapted" for intraperitoneal growth.

The evidence of Algire *et al.* (1–3) is in opposition to the hypothesis that tissue within the Millipore might produce a degree of tolerance in the host. However, their studies were carried out in crosses between inbred strains of mice, and with mouse-rat heterotransplants; this is in contrast to the present experiments, which were performed in the albino rat. This may be an important difference, for the inbreeding in commercial rat colonies apparently produces a slight degree of natural tolerance to homotransplanted tissues. For instance, Ashley *et al.* (17) have found a permanent success of intra-strain skin homografts in about 10% of young rats; and although many authors (18–23) have reported the failure of all attempts to secure successful skin homografts in the normal untreated adult rat, in our laboratory a few skin homografts in adult rats have been observed to live as long as 66 days. The "partial homograft tolerance" of the rat has also been

demonstrated by the prolonged survival of skin homotransplants performed by McQuarrie *et al.* (24).

An additional question of great importance deals with the problem of assessment of function of the thyroid tissue in the Millipore chambers. Although this tissue has been shown to pick up I^{131} in the area of the follicles, it must in addition be demonstrated that the inorganic iodide is incorporated into thyroxine. This problem is currently under investigation.

Acknowledgments

The authors are indebted to Dr. Gustave Dammin for the invaluable assistance which he and other members of the Department of Pathology at the Peter Bent Brigham Hospital have provided. The careful technical work of Nancy Metcalfe and Nancy O'Donahue is also gratefully acknowledged.

REFERENCES

1. Algire, G., J. M. Weaver and R. T. Prehn: *J. Nat. Cancer Inst.* **15:** 439. 1954.
2. Prehn, R., J. M. Weaver and G. H. Algire: *J. Nat. Cancer Inst.* **15:** 509. 1954.
3. Weaver, J., G. H. Algire and R. T. Prehn: *J. Nat. Cancer Inst.* **15:** 1737. 1955.
4. Amos, D. and J. D. Wakefield: *J. Nat. Cancer Inst.* **21:** 657. 1958.
5. Duthie, R. B.: *Brit. J. Plast. Surg.* **11:** 1030. 1958.
6. Grobstein, C.: *Exper. Cell Research* **10:** 424. 1956.
7. Sturgis, S. and H. Castellanos: *Proc. Soc. Exper. Biol. & Med.* **94:** 569. 1957.
8. Sturgis, S. and H. Castellanos: *Am. J. Obst. & Gynec.* **76:** 1132. 1958.
9. Jordan, G., Jr., R. P. Foster and F. Gyorkey: *Plast. & Reconstruct. Surg.* **22:** 392. 1958.
10. Stone, J. and J. F. Ehring, Jr.: *Transplantation Bulletin* **4:** 17. 1957.
11. Hallin, R. and H. Swan: *Surgical Forum* **IX:** 628. 1958.
12. Duthie, R., R. M. Merwin and J. Wolff: *Exper. Cell Research* **16:** 565. 1959.
13. Brooks, J., J. C. Priario and A. de'Scoville: *Surgical Forum* **IX:** 633. 1958.
14. Anon: "Millipore Filters. 1958 Supplement" Millipore Filter Corp., Watertown, Mass.
15. Parker, R. and J. F. Morgan. *Methods of Tissue Culture.* P.10. Paul Hoeber, New York, 1950.
16. Koprowski, H.: *Fed. Proc.* **16:** 592. 1957.
17. Ashley, F., R. F. Swan, A. N. Schwartz, G. Joseph, H. Stein and W. P. Longmire, Jr.: *Plast. and Reconstruct. Surg.* **22:** 462. 1958.
18. Lehrfeld, J., A. C. Taylor and J. M. Converse: *Plast. and Reconstruct. Surg.* **15:** 74. 1955.
19. Woodruff, M. and L. O. Simpson: *Plast. & Reconstruct. Surg.* **15:** 451. 1955.
20. Ballantyne, D. and J. M. Converse: *Ann. New York Acad. Sc.* **64:** 958. 1957.
21. Woodruff, M.: *Ann. New York Acad. Sc.* **64:** 1014. 1957.
22. Levinson, M. and H. Necheles: *Plast. & Reconstruct. Surg.* **17:** 218. 1956.
23. Ashley, F., H. Stein, R. Peterson, F. Grazer and W. P. Longmire, Jr.: *Transplantation Bulletin* **5:** 29. 1958.
24. McQuarrie, D., J. H. Kim and R. L. Varco: *Transplantation Bulletin* **5:** 97. 1959.

THE MASS OF THE ZONA GLOMERULOSA FOLLOWING COMPLETE ANTERIOR AND POSTERIOR HYPOPHYSECTOMY AND SUBTOTAL REMOVAL OF THE PARS TUBERALIS IN THE DOG[1]

ROBERT C. BAHN, HENRY E. STORINO AND ROBERT W. SCHMIT

Mayo Clinic and Mayo Foundation, Rochester, Minnesota

ABSTRACT

Atrophy of both the zona glomerulosa and the inner adrenal cortical zones of the dog was observed 6 weeks after complete anterior and posterior hypophysectomy and subtotal removal of the pars tuberalis. Reimplantation of a portion of the pars distalis resulted in partial maintenance of the mass of each of the adrenal cortical zones. The degree of atrophy of the zona glomerulosa was less than that observed in the inner cortical zones. Nevertheless, the data demonstrate that in the dog the mass of the zona glomerulosa is not exclusively controlled by extrapituitary factors.

BECAUSE of the rounded configuration of the adrenal gland, evaluation of the effects of hypophysectomy on the cells of the zona glomerulosa has been difficult. Histologic sections of the adrenal are usually prepared in such a way that the cross section of the gland appears almost elliptical or circular. After hypophysectomy the inner cortical zones (zona fasciculata and zona reticularis) promptly decrease in size. In evaluating the effects of hypophysectomy on the morphology of the zona glomerulosa by means of microtome sections, one is faced with the problem of estimating the total mass of a population of cells distributed about the circumference of a shrinking cylinder or sphere (1, 2). Since marked atrophy of the inner cortical zones might be expected to minimize or obliterate changes in the thickness of the zona glomerulosa resulting from hypophysectomy, one would not expect measurements of only the thickness of the zona glomerulosa to be a reliable index of the latter's total mass (2). The method of Erankö (3) for estimation of the volume of the cortex and medulla of the rat's adrenal necessitates complete serial sections of the adrenal and therfore seems impractical when applied to studies of substantial numbers of dogs' adrenals. The method developed for the present study is based on the removal of an almost cylindrical portion of the dog's adrenal gland from a region where the cortex is usually uniform in composition and thickness.

Received July 17, 1959.

[1] This work was supported in part by research grant no. A-1944 from the Public Health Service.

The thicknesses of the cortical zones within this sample and the cross sectional area of each zone within the sample were determined. From these data and the weights of the adrenal glands, the mass of the various layers of the adrenal cortex was calculated.

MATERIALS AND METHODS

Experimental Plan. Fifteen mongrel dogs were studied. All dogs were maintained on the usual laboratory rations of dried pellets or canned dog food. Normal preoperative intake of food was maintained in hypophysectomized dogs during the postoperative period. Water was given as desired.

The animals were divided into three groups. Group 1 consisted of six normal dogs, each weighing approximately 20 kg. The mean total weight for pairs of adrenals was $2.08 \pm .05$ gm. (mean $\pm$ standard error of the mean).

Group 2 consisted of six totally hypophysectomized dogs. Hypophysectomy was performed through a left temporal approach. The anterior pituitary gland was visualized directly and removed together with the anterior half of the median eminence. All dogs developed mild permanent diabetes insipidus as well as pituitary insufficiency and were sacrificed 6 weeks postoperatively. The mean initial body weight was 14.1 kg., the mean final body weight was 13.2 kg., and the mean total weight for pairs of adrenals was 0.74 ± 0.05 gm.

Group 3 consisted of three hypophysectomized dogs in which half of the pars distalis was reimplanted into the sella turcica. Immediately after removal of the pituitary and placement of the lesion in the median eminence as previously described, the freshly removed pars distalis was dissected free from other portions of the hypophysis and divided in half, and one of these two portions was immediately replaced into the sella. Dogs of group 3 were sacrificed 6 weeks postoperatively. The mean initial body weight was 13.5 kg. The mean final body weight was 12.6 kg. The mean total weight for pairs of adrenals was 1.02 ± 0.13 gm.

All adrenal glands were fixed in 10 per cent formalin solution. After fixation the extra adrenal fat and connective tissue were carefully trimmed away and the glands weighed. Identification of reimplanted pars distalis, localization of the median-eminence lesions, and verification of the completeness of the hypophysectomy were determined by serial sections of the hypothalamus and contents of the sella turcica.

Sampling of Tissue. An almost cylindrical sample of the tissue approximately 3 to 4 mm. in thickness was selected from the peninsular process of the right adrenal gland, near the junction of the latter with the body of the adrenal. The base of the peninsular process of the right adrenal was selected because this site could easily be identified and an almost circular or elliptical cross section possessing few major irregularities in the cortical layers could be obtained from most pairs of adrenal glands. Frozen microtome sections were prepared from this sample and stained with hematoxylin and Sudan IV.

Measurement of Cortical Zones and Medulla. Ten measurements of the thickness of the zona glomerulosa and 10 measurements of the sum of the thicknesses of the inner cortical zones (zona fasciculata and zona reticularis) were made about the circumference of each frozen section by means of a graduated ocular micrometer at a magnification of 32×. The semimajor and semiminor axes of the medulla were determined by measuring the major and minor axes and dividing each of these values by two.

Calculation of Cross Sectional Areas of Zones Within Sampled Section. The sampled adrenal section was considered to be composed of a series of three concentric ellipses the limits of which were defined by (a) the outer margin of the medulla, (b) the outer margin of the zona fasciculata and (c) the outer margin of the zona glomerulosa. The semimajor axis of the sampled section was found by adding the length of the semimajo

axis of the medulla to the sum of the mean thicknesses of the cortical zones. The semiminor axis of the sampled section was found by adding the length of the semiminor axis of the medulla to the sum of the mean thicknesses of the cortical zones. The semiaxes of the ellipse bounded by the outer margin of the zona fasciculata were computed in a similar manner. The cross sectional area of each concentric ellipse within a section was computed by multiplying the appropriate semimajor axis by the appropriate semiminor axis and then multiplying this product by π. The cross sectional area of each individual zone was derived by subtracting the area of one ellipse from the area of the next largest ellipse.

Calculation of Mass of Adrenal Cortical Zones. The total mass of a zone (M_z) was calculated from the weight of the adrenal (M_t), the total area of the sampled section (a_t) and the area of the zone within the sampled section (a_z) by means of the following formula:

$$M_z = \frac{(a_z)(M_t)}{a_t}.$$

The derivation of this relationship is presented in the appendix. The means, the standard errors of the means and the value for t were determined in the usual manner.

RESULTS

The results and statistical comparisons are listed in Tables 1 and 2. After total hypophysectomy (group 2) there was a major decrease in both the thickness and the mass of the inner cortical zones. This atrophy was partially prevented by reimplantation of the pars distalis (group 3).

Although the thickness of the zona glomerulosa was greater after total hypophysectomy (groups 2 and 3) the observed differences were not statistically significant. The mass of the zona glomerulosa, on the other hand, decreased significantly after total hypophysectomy (group 1 versus group 2, $P < .05$). However, atrophy of the zona glomerulosa was not demonstrable in hypophysectomized dogs in which a portion of the pars distalis was replaced into the sella turcica (group 3).

Differences in the mass of the medulla among groups 1, 2 and 3 were not significant.

TABLE 1. EFFECTS OF COMPLETE HYPOPHYSECTOMY ON THICKNESS OF ADRENAL CORTICAL ZONES

Animals		Hypophysis and Hypothalamus				Adrenal thickness, mm.	
Group	Number	Pars distalis	Pars tuberalis	Pars nervosa	Ant. med. eminence	Z. glom.	Inner Z.
1 (Normal)	6	Intact	Intact	Intact	Intact	.22 ± .03*	1.54 ± .08
2 (Hypophysectomized)	6	Absent	Minute remnants	Absent	Lesion	.27 ± .001	.64 ± .10
3 (Hypophysectomized, with replacement)	3	Small fragment (15 to 30%)	Minute remnants	Absent	Lesion	.25 ± .004	1.13 ± .09
1 versus 2						t = 1.56 P > .05	t = 6.92 P < .01
2 versus 3						t = 1.42 P > .05	t = 3.06 P < .05
1 versus 3						t = 0.63 P > .05	t = 3.15 P < .05

* Mean and standard error of the mean.

TABLE 2. EFFECTS OF COMPLETE HYPOPHYSECTOMY ON MASS OF ADRENAL CORTICAL ZONES

Animals		Mass, mg./kg. body weight		
Group	Number	Z. glom.	Inner Z.	Medulla
1 (Normal)	6	15.5±0.9*	72.5±1.9	16.1±1.4
2 (Hypophysectomized)	6	11.7±1.2	21.7±2.3	23.4±3.5
3 (Hypophysectomized, with replacement)	3	14.5±1.9	48.1±7.1	18.2±6.1
1 versus 2		t=2.55 P <.05	t=19.0 P <.01	t=1.96 P >.05
2 versus 3		t=1.33 P >.05	t=4.60 P <.01	t=0.61 P >.05
1 versus 3		t= .56 P >.05	t=4.57 P <.01	t=0.97 P >.05

* Mean and standard error of the mean.

COMMENT

A number of authors (4, 5, 6, 7, 8) have reported that after hypophysectomy subtotal maintenance of the morphology and function of the dog's adrenal cortex has been associated with the persistence of minute remnants of the pars distalis with the sella. Marked adrenal cortical atrophy was a result of total hypophysectomy.

Lane and de Bodo (7) have reported that atrophy of the zona glomerulosa as well as of the inner adrenal cortical zones can be observed 6 to 18 months after complete hypophysectomy in the dog. In the present study significant atrophy of both the zona glomerulosa and the inner cortical zones followed combined complete anterior and posterior hypophysectomy and subtotal removal of the pars tuberalis. These data seem to indicate that trophic activities affecting the zona glomerulosa as well as the inner cortical zones arise from the region of the median eminence and the pituitary gland of the dog. This conclusion is supported by the observation of partial maintenance of the mass of all the adrenal cortical zones by replacement of a portion of the pars distalis into the sella turcica. The importance of the anterior pituitary and the tuberal region in the control of aldosterone secretion has recently been discussed by Davis and associates (9, 10, 11).

Our data also show a distinct difference between the degree of atrophy observed in the zona glomerulosa and that observed in the inner cortical zones after complete hypophysectomy (group 2). This disparity suggests that some factors other than the pars distalis play a role in the maintenance of the mass of the zona glomerulosa 6 weeks after hypophysectomy. The extrapituitary factors have been investigated (12, 13) and reviewed (14) by Farrell and associates.

Acknowledgment

The authors wish to thank Dr. James O. Davis, National Heart Institute, Bethesda, Maryland, for supplying the adrenal glands of normal dogs used in this study. Dr. Davis' advice and criticism of this work were greatly appreciated.

REFERENCES

1. Greep, R. O. and H. W. Deane: *Ann. New York Acad. Sc.* **50:** 596. 1949.
2. Knobil, E., A. Morse, F. G. Hofmann and R. O. Greep: *Acta endocrinol.* **17:** 229. 1954.
3. Erankö, O.: *Ann. med. exper. et biol. Fenniae* **32:** 377. 1954.
4. Keller, A. D., W. E. Lawrence and C. B. Blair: *Arch. Path.* **40:** 289. 1945.
5. Goodkind, M. J., J. O. Davis, W. C. Ball, Jr. and R. C. Bahn: *Am. J. Physiol.* **188:** 529. 1957.
6. Eik-Nes, K. and K. R. Brizzee: *Am. J. Physiol.* **193:** 403. 1958.
7. Lane, N. and R. C. de Bodo: *Am. J. Physiol.* **168:** 1. 1952.
8. Keller, A. D. and C. G. Breckenridge: *Am. J. Physiol.* **150:** 222. 1947.
9. Davis, J. O., R. C. Bahn, N. A. Yankopoulos, B. Kliman and R. E. Peterson: *Am. J. Physiol.* **197:** 380. 1959.
10. Davis, J. O., R. C. Bahn and W. C. Ball, Jr.: *Am. J. Physiol.* **197:** 387. 1959.
11. Davis, J. O., N. A. Yankopoulos, J. Holman and R. C. Bahn: *Physiologist* **2:** 29. 1959.
12. Newman, A. E., E. S. Redgate and G. Farrell: *Endocrinology* **63:** 723. 1958.
13. Farrell, G., S. Koletsky and L. W. Lapham: *Fed. Proc.* **18:** 44. 1959.
14. Farrell, G.: *Physiol. Rev.* **38:** 709. 1958.
15. Goodsell, J. E.: *Am. J. Physiol.* **134:** 119. 1941.
16. Baker, D. D.: *Am. J. Anat.* **60:** 231. 1937.

APPENDIX

The method for the calculation of the mass of the adrenal zones is based upon the following considerations: If any solid figure is divided by a sufficiently large number $(n-1)$ of parallel planes at sufficiently small equal intervals (t), a large number (n) of figures will result which possess an area (A) parallel to the plane of section, and a thickness (t). The total volume (V_t) of the solid figure can be expressed as:

$$V_t = A_1t + A_2t + A_3t + \cdots + A_nt \tag{1}$$

or

$$V_t = t(\Sigma A) \tag{2}$$

Multiplying the right hand member by n/n

$$V_t = nt\left(\frac{\Sigma A}{n}\right) \tag{3}$$

If

$$nt = s \tag{4}$$

and $\Sigma A/n = \bar{A}t$ where $\bar{A}_t$ is the mean cross sectional area, then

$$V_t = s\bar{A}_t \tag{5}$$

A similar expression can be derived for the volume of a zone (V_z) within the solid figure in terms of the mean cross sectional area of the zone $(\bar{A}_z)$, thus

$$V_z = s\bar{A}_z \tag{6}$$

The mass (M_t) of a solid figure can be defined in terms of its volume (V_t) and its density (k_t) as:

$$M_T = k_t V_t \tag{7}$$

or

$$V_t = \frac{1}{k_t} M_t \tag{8}$$

Similarly the volume of a zone within the solid figure (V_z) can be defined in terms of its mass (M_z) and density (k_z) as:

$$V_z = \frac{1}{k_z} M_z \tag{9}$$

Substituting for V and dividing equation 6 by equation 5,

$$\frac{k_t}{k_z} \frac{M_z}{M_t} = \frac{s\bar{A}_z}{s\bar{A}_t} \tag{10}$$

Solving for M_z

$$M_z = \frac{k_z}{k_t} \frac{\bar{A}_z M_t}{\bar{A}_t} \tag{11}$$

If one assumes that the ratio of the area of a zone within the sample (a_z) to the total area of the sampled section (a_t) approximates the ratio of the true mean areas $\bar{A}_z$ and $\bar{A}_t$ and that there are no appreciable differences in the densities of the layers of the fixed hydrated adrenal gland, then

$$k_z = k_t \tag{12}$$

and

$$\frac{a_z}{a_t} = \frac{\bar{A}_z}{\bar{A}_t} \tag{13}$$

Substituting equations 12 and 13 in equation 11,

$$M_z = \frac{a_z}{a_t} M_t \tag{14}$$

for ease of calculation

$$\frac{M_t}{a_t} = K \tag{15}$$

Substituting K in equation 14,

$$M_z = K a_z \tag{16}$$

The assumption that the ratio a_z/a_t approximates the ratio $\bar{A}_z/\bar{A}_t$ seems justified since the relative composition of the adrenal gland of normal adult dogs calculated by our methods (zona glomerulosa 16 per cent, inner cortical zones 69 per cent, medulla 15 per cent) does not differ remarkably from that derived from gross dissection of the adrenal cortex and medulla (15) and from serial sections of the adrenal gland (16).

EFFECTS OF VARIOUS ANALOGUES OF THYROXINE ON OXYGEN UPTAKE OF CARDIAC MUSCLE FROM CHICKS[1]

W. S. NEWCOMER AND PAUL A. BARRETT[2]

Department of Physiology and Pharmacology, Oklahoma State University, Stillwater, Oklahoma

ABSTRACT

The response in oxygen uptake of surviving ventricular myocardium to thyroxine, 3:5:3′-triiodothyronine, 3:5-diiodothyronine and 3-monoiodothyronine and their acetic and propionic acid derivatives was studied in cockerels pre-treated with thiouracil. Thiouracil lowered the oxygen uptake from the normal control level of 6.0 μl./hr./mg. dry tissue to 4.0 μl. Thyroxine gave a greater increase above the normal controls than any of the other compounds at all dosage levels 12 hours following intraperitoneal injection; at the higher dosage levels, triiodothyronine was the next most potent compound. The remaining compounds, in general, did not increase oxygen uptake above the normal control level except at the highest dose. The response to thyroxine reached a maximum at 9–12 hours and subsided by 36 hours; that to triiodothyronine at 12–15 hours and 18 hours respectively.

ALTHOUGH triiodothyronine has been identified in the thyroid glands (1, 2) and blood of normal chickens (3), the physiologic significance of this analogue of thyroxine within the thyroid or at the peripheral level remains unknown in the bird. Triiodothyronine has been reported to have less potency than thyroxine in blocking TSH release (4), the same potency in preventing goiter (5) and controlling suffocation time, total oxygen consumption and heart rate (6), and greater potency in preventing goiter in thiouracil-treated birds (6, 7). In view of these discrepancies in relative activities and the lack of information concerning the possible biologic roles of any analogues of thyroxine at the peripheral level in the bird, the effects of thyroxine and some of its derivatives on oxygen uptake of cardiac muscle were studied in the chick.

MATERIALS AND METHODS

White Leghorn cockerels were obtained from Stillwater Hatchery on the day of hatching and were placed in a battery chick brooder where they received "Superior All-In-One" chicken feed and 0.1% thiouracil in their drinking water for 7–14 days. At the desired time prior to sacrifice, each chick received an intraperitoneal injection of one of the following 12 compounds (all levorotatory forms): thyroxine (T_4), 3:5:3′-

Received August 5, 1959.

[1] Supported by a grant from the Oklahoma State Heart Association.

[2] Submitted in part as a thesis by the junior author for the M.S. degree.

triiodothyronine (T_3), 3:5-diiodothyronine (T_2) and 3-monoiodothyronine (T_1) and their acetic and propionic acid derivatives (T_4A, T_4P, T_3A, etc.). T_4 was given in logarithmic doses of 0.01, 0.1, 1, 10, and 100 μg./100 gm. of body weight. The other 11 compounds were given on a molar equivalent basis as compared to T_4 (Fig. 1). Each of the 12 compounds was dissolved in such a manner that all injections were 0.1 ml./100 gm. body weight. All of the 12 compounds used except T_4P and T_4A were dissolved in a weak solution of NaOH and then diluted to the desired volume with distilled water. T_4P and T_4A lose their potency if mixed in weak NaOH solution (8); therefore they were dissolved in 1 part ethanol and diluted with 9 parts propylene glycol. The solutions prepared for injection were refrigerated in order to minimize deterioration of the compounds in alkaline medium; unused solutions were discarded at the end of 3 days.

The chicks were killed by decapitation and the ventricular myocardium removed and placed in an ice-cold phosphate saline solution which had been prepared and refrigerated 2 hours previously (9). The myocardium was cut into uniform slices of 0.5 mm. in thickness by means of a Stadie-Riggs microtome. Two or three slices of tissue from the same heart were placed in a Warburg vessel which contained 3 ml. cold phosphate saline solution. The vessel was mounted, flushed with pure oxygen for 5 mintues, then allowed to equilibrate in the water bath for 15 minutes at 37° C. Oxygen uptake readings were taken at 10 minute intervals for 1 hour at a constant temperature of 37° C. At the end of one hour, the slices of myocardium were removed and dried at 37° C for 48 hours. Oxygen uptake was calculated as μl. O_2/mg. dry tissue/hr.

Since cardiac tissue from only 20 chicks could be handled in the Warburg apparatus at one time, one or two birds were used at each dosage level along with one normal control and one thiouracil control bird for each "run" in order to minimize experimental variation between dosage groups.

Since the regression of the plotted data involved different levels of a single factor (Fig. 1) and since the experiment was completely randomized, orthogonal comparisons in regression were made utilizing Snedecor's "F" test to obtain levels of probability (10).

RESULTS

The responses of ventricular myocardium from chicks to various levels of thyroxine and 11 of its analogues, as measured by oxygen uptake of surviving tissue slices 12 hours following injection, are shown in Figure 1.

All of the compounds tested increased the oxygen consumption of cardiac muscle above the level of oxygen uptake of myocardium from the thiouracil-treated control birds; this increase was obtained at all dosage levels. The responses obtained from T_4 were greater than those of any of the other compounds at all levels. The regression line for T_3 indicated the response to T_3 was in general greater than those of the other compounds (except T_4) at levels equivalent to 1 to 100 μg. T_4. Of the remaining derivatives of thyroxine used in this study, only T_3A, T_3P, T_2, T_2P, T_1 and T_1P elevated the oxygen uptake above that of the normal controls and then only at the highest dosage levels. Thiouracil treatment alone for 7–14 days reduced the oxygen uptake of surviving ventricular slices from the untreated control level of $6.0 \pm .3$ (40 birds) to $4.0 \pm .38$ (37 birds) μl./hr./mg. dry tissue. Statistical analysis (orthogonal comparison in regression) indicated that the response to many of the compounds contained a linear component which was statistically significant ($P < .01$ for T_4, T_3, T_3P, T_2P, and T_3A

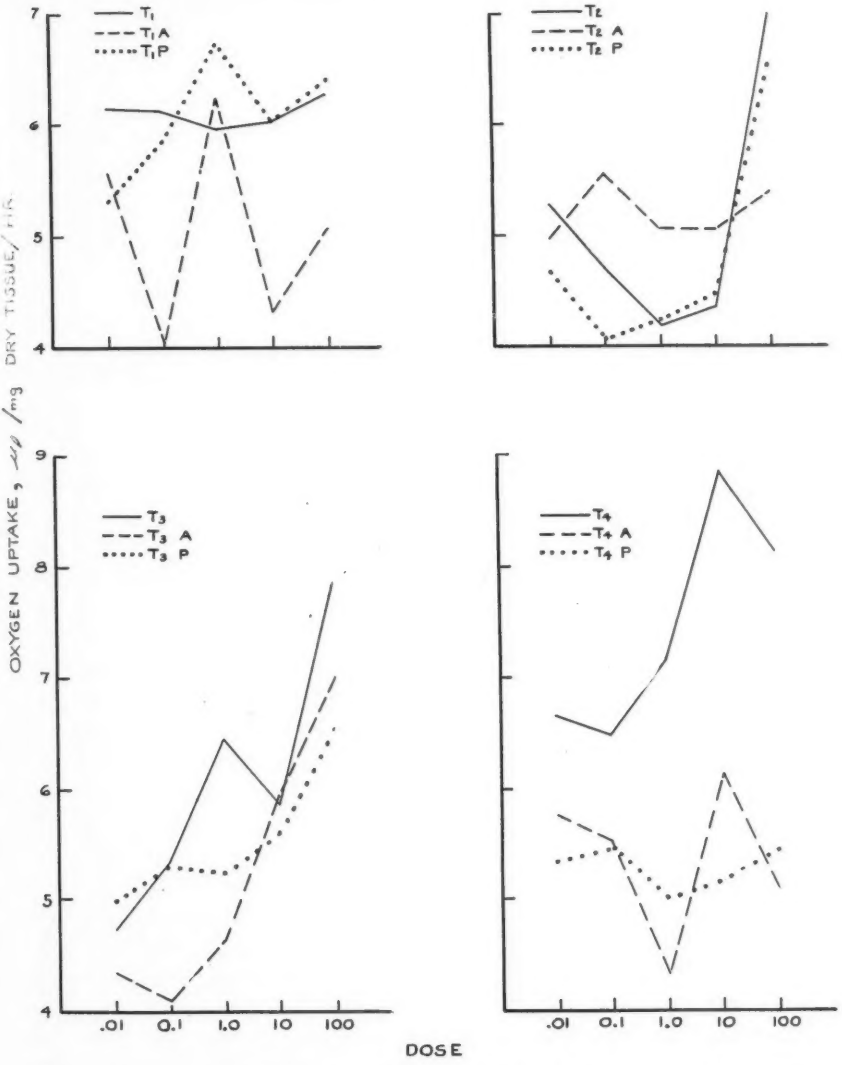


FIG. 1. Responses of cardiac muscle to various doses of thyroxine and some of its analogues. O_2 uptake of myocardium from untreated control birds was $6.0 \pm .3$ μl/hr./mg. dry tissue.

$P < .05$ for T_2). Other components (quadratic, cubic and quartic) with a high level of significance were also obtained.

These results indicate that 12 hours following injection of the hormone, T_4 stimulated a greater increase in oxygen uptake of cardiac muscle from the chick than did T_3 or any of the other derivatives following equivalent

TABLE 1. OXYGEN UPTAKE* AT VARIOUS INTERVALS AFTER INJECTION OF T_4† AND MOLAR EQUIVALENT AMOUNTS OF T_3, T_3A, T_3P AND T_4A

Treatment		Hours after injection									
		3	6	9	12	15	18	24	30	36	42
T_4	Number	10	10	20	20	20	10	10	10	10	10
	Mean	3.28	4.89	9.63	8.56	7.95	7.05	5.84	4.63	3.95	4.60
	St. Dev.	.7	1.39	2.0	1.1	2.4	1.7	1.5	.6	.9	1.4
T_3	Number	10	10	10	20	20	10	10	10	10	10
	Mean	4.06	4.16	6.14	5.97	7.79	4.17	3.53	3.71	3.75	3.73
	St. Dev.	1.1	1.3	1.1	1.1	3.0	.98	.8	.78	.1	.5
T_3P	Number	10	9		10						
	Mean	5.82	6.30		5.61						
	St. Dev.	1.0	.7		1.57						
T_3A	Number	10	10		10						
	Mean	5.71	5.43		5.94						
	St. Dev.	.9	1.2		1.0						
T_4A	Number	10	9		10						
	Mean	3.73	4.49		6.13						
	St. Dev.	.5	1.09		2.07						

* μl./hr./mg. dry tissue.
† 10 μg./100 gm. body weight.

doses. In general, all of the other compounds tested were less potent than T_3 under similar experimental conditions.

When the dose of hormone was constant at 10 μg./100 gm. body weight (or its equivalent) and the time interval was varied, the maximal response to T_4 occurred at 9 hours following injection (Table 1); that to T_3 occurred at 15 hours. The increased oxygen uptake following injection of T_4 did not return to the thiouracil control level until 36 hours following injection; that of T_3 returned at 18 hours. Integration of response and time by use of a planimeter gave values of 7.0 units for T_4 and 2.6 units for T_3. These data indicate that the response of increased oxygen uptake by chick cardiac muscle to T_4 was quicker, greater in magnitude, and more prolonged than that of T_3 under the conditions of this experiment.

DISCUSSION

In spite of numerous reports on the biologic effects of various thyronines following the identification of T_3 in the blood of human beings (11) and knowledge of its wide distribution in mammals, there is no general agreement on the significance of their physiologic actions or interrelations either within the thyroid gland or at the tissue level. T_3 is credited with being more potent than T_4 as evidenced by measurements of numerous criteria of action in mammals (12); T_3, T_4A, or T_3A were regarded by different workers as being the peripherally acting thyroid hormone (13, 14, 15).

Although relative potencies can be evaluated by different standards, the term as used herein refers to a comparison of responses obtained with molar equivalent amounts of two (or more) substances respectively. The results of this study indicate that T_4 is more potent than T_3 in the chick when two different standards of potency are utilized. T_4 showed greater activity than did an equimolar amount of T_3 in increasing oxygen uptake of surviving

ventricular slices 12 hours following injection of the hormones at five different dosage levels into chicks treated with thiouracil (Fig. 1). T_4 was also the more potent compound when potency was measured by the integration of duration and magnitude of response to molar equivalent amounts of the two compounds (Table 1). These results are in agreement with those of Newcomer (6) and Mellen and Wentworth (7) who reported T_4 to be more potent than T_3 in preventing goiter in thiouracil-treated birds.

The maximal response to 10 μg. T_4 (per 100 gm. body weight) was obtained 9 hours after injection; that to T_3, 15 hours after injection of an equimolar amount. Due to the variation within groups of birds at points adjacent to the peaks and due to the general shapes of the respective response curves, it is more judicious to express the maximal response as occurring within a time interval of 9–12 hours for T_4 and 12–15 hours for T_3. The decision to compare all the analogues of thyroxine at 12 hours was based on this justification. The response to T_4 was therefore quicker and of longer duration than that to T_3. The relatively rapid appearance and short duration of the response of cardiac muscle of the chick to T_4 as compared with the mammal is in agreement with the findings of Mellen (16) who reported the stimulating effects of T_4 on BMR to persist for only a few (13–18) hours after withdrawal of the hormone (in feed). As is indicated in Table 1, the response of cardiac muscle to T_4 subsided by approximately 36 hours following injection and that to T_3 by 18 hours.

The physiologic reason(s) for the reversal of relative potencies of T_4 and T_3 in birds as compared to mammals (12), or as compared to results utilizing other criteria of hormonal action such as TSH release in birds (4), is not evident due to the lack of information concerning the actions and metabolism of thyroid hormones in the bird. The reason probably is not due to a difference in plasma protein-binding, catabolism or excretion, since Tata and Shellabarger (17) reported the biological half-lives of both T_3 and T_4 to be 22.5 hours and the plasma protein binding affinities to be the same. Neither are the different potencies due to number of binding sites on the target organ (heart tissue) or affinity of hormone to binding sites as evidenced by an application of the data obtained in this study to a formula derived by Stetten in analogy to that of the Michaelis-Menton concept (20). When the dose of hormone employed/response in oxygen uptake of cardiac muscle was plotted against dose, the regression curves for T_4 and T_3 were practically identical straight lines.

An interesting result of this study is that all of the compounds tested exhibited some activity above that of the thiouracil-treated birds. Since the relative potencies of the compounds other than T_4 and T_3 are based only on a time interval of 12 hours following injection, it is entirely possible that some of these derivatives would have stimulated a greater oxygen uptake had they been tested at other time intervals. Special interest in this regard is associated with T_3, T_3A and T_4A which have been suggested as being

peripheral hormones in mammals (13, 14, 15). The responses of oxygen uptake 3 and 6 hours following injection of T_3A, T_3P and T_4A respectively are found in Table 1. In each case the response obtained was greater than that due to either T_3 or T_4 at the same time interval; the responses were not substantially different from those at 12 hours. This relatively rapid response to these compounds as compared to T_4 or T_3 is certainly not sufficient evidence in itself for regarding any one of these substances as necessarily being a peripheral hormone in the bird to the exclusion of any of the others. The evidence does indicate the relatively nonspecific nature of the cardiac receptor sites.

Of further interest in the comparison of the effects of the various analogues on oxygen uptake of cardiac muscle is the form of the dose-response curve for each compound. In many of the cases the response was linearly proportional to dose; the responses to T_4A, T_4P, T_2A, T_1 and T_1A were not proportional to dose. The physiologic reason(s) for the forms (quadratic, cubic and quartic) of the dose-response curves which were not linear is not evident from the available data although in a number of instances the forms of the curves were statistically significant.

In addition to information concerning relative potencies (which involves a comparison of the responses of one target organ to two or more different substances), this study offers a basis of comparison of the relative sensitivity of cardiac muscle to T_4 with other target organs in the chick. By relative sensitivities is meant a comparison of responses of two (or more) target organs by biologically equivalent doses such as the amount of hormone necessary to maintain normal control value of response in two different target organs; this comparison could involve dissimilar units of response.

The fact that 0.01 μg. T_4/100 gm. body weight stimulated the oxygen uptake of ventricle slices above the value for normal controls indicates that the heart is very sensitive to small amounts of this hormone. This sensitivity is more impressive when compared to that of inhibition of TSH release from the pituitary as in the Dempsey-Astwood technique which has been frequently utilized in estimating T_4 secretion rate. If a secretion rate of 2.45 to 2.9 μg. T_4/100 gm. body weight by the Dempsey-Astwood method (16, 18) is taken as a representative figure for chicks of the age employed in this study, and if it is assumed that the injection of T_4 would have to be made every 12 hours to maintain an oxygen uptake equal to that of the normal controls, the sensitivity of the heart to T_4 is over 100 times greater than that of the pituitary-thyroid axis. While there is some inconsistency in making this comparison due to different experimental procedure, this difference supports the suggestion of Newcomer (6) and Van Middlesworth *et al.* (19) that the Dempsey-Astwood technique could give an erroneously high value for the T_4 secretion rate. These data, however, should not be construed to suggest that the thyroxine secretion rate is a few hundredths μg./day either. A final answer to the problem of what criterion of hormonal

action to choose in estimating T_4 secretion rate is difficult since the choice involves many variables such as dose, time, number of receptor sites on the target, affinity of receptor sites for the hormone, number of biochemical steps involved in "producing" the criterion to be measured, "turnover" rate and/or inactivation of hormone molecules at the target organ. The molecular action of T_4 at the cellular level remains to be demonstrated; it is possible that its action might not be the same at all target organs which it affects.

Acknowledgments

We wish to thank Smith, Kline, and French Laboratories for generously providing T_3 and Warner-Lambert Research Institute for all the remaining compounds except T_4 and T_2 which were purchased from Sigma Chemical Company. We are grateful to Dr. R. Morrison for counsel with statistics.

REFERENCES

1. Shellabarger, C. J. and R. Pitt-Rivers: *Nature* **181:** 546. 1958.
2. Frey, H. and E. V. Flock: *Acta Endocrinol.* **29:** 550. 1958.
3. Mellen, W. J. and B. C. Wentworth: Personal Communication.
4. Gilliland, I. C. and J. I. Strudwick: *Memoirs Society Endocrinology* **1:** 14. 1953.
5. Shellabarger, C. J.: *Poult. Sci.* **34:** 1437. 1955.
6. Newcomer, W. S.: *Am. J. Physiol.* **190:** 413. 1957.
7. Mellen, W. J. and B. C. Wentworth: *Poult. Sci.* **38:** 228. 1959.
8. Stasilli, N. R., R. L. Kroc and R. I. Meltzer: *Endocrinology* **64:** 62. 1959.
9. Krebs, H. A. and L. V. Eggleston: *Biochem. J.* **34:** 442. 1940.
10. Snedecor, G. W.: *Statistical Methods*, Iowa State College Press, Ames, Iowa. 1956.
11. Gross, J. and R. Pitt-Rivers: *Lancet*, 1, **439:** 593. 1952.
12. Barker, S. B.: *Ann. Rev. Physiol.* **17:** 417. 1955.
13. Gross, J. and R. Pitt-Rivers: *Biochem. J.* **53:** 645. 1953.
14. Thibault, O. and R. Pitt-Rivers: *Lancet* **268:** 285. 1955.
15. Thibault, O. and R. Pitt-Rivers: *C. R. Soc. Biol., Paris* **149:** 880. 1955.
16. Mellen, W. J.: *Poult. Sci.* **37:** 672. 1958.
17. Tata, J. R. and C. J. Shellabarger: *Biochem. J.* **71:** 3P. 1959.
18. Reineke, E. P. and C. W. Turner: *Mo. Agr. Exper. Sta. Res. Bull.* **392.** 1945.
19. Van Middlesworth, L., G. Jagiello and W. P. Vanderlaan: *Endocrinology* **64:** 186. 1959.
20. Stetten, D.: *Science* **124:** 365. 1956.
21. Klitgaard, H. M. and R. A. Whaley: *Physiologist* **1:** 50. 1957.
22. Barker, S. B. and H. M. Klitgaard: *Am. J. Physiol.* **170:** 81. 1952.

19-HYDROXY-Δ^4-ANDROSTENE-3,17-DIONE AN INTERMEDIATE IN ESTROGEN BIOSYNTHESIS[1]

JACQUES E. LONGCHAMPT,[2] CARLOS GUAL, MAXIMILIAN EHRENSTEIN AND RALPH I. DORFMAN

Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts and Division of Steroid Research, Department of Research Medicine, University of Pennsylvania, Philadelphia, Pennsylvania

ABSTRACT

Incubation of Δ^4-androstene-3,17-dione-4-C^{14} with a human placental preparation yielded 19-hydroxy-Δ^4-androstene-3,17-C^{14} in addition to estrone-C^{14}. The 19-hydroxy steroid had previously been shown to be converted in good yield to estrone. This is the first direct demonstration of 19-hydroxylase in a tissue other than adrenal and more firmly establishes the 19-hydroxy derivative as an intermediate in the biosynthesis of estrogens from androgens.

IN THE course of studies on the pathways of estrogen biosynthesis from testosterone and Δ^4-androstene-3,17-dione a substance with the chemical and physical properties of 19-hydroxy-Δ^4-androstene-3,17-dione was observed in our laboratory and by Ryan (2, 17). This substance, a logical intermediate, has been shown to be converted to estrogens in good yield in steroid producing tissues by Meyer (3), Ryan (2, 17) and Gual *et al.* (1). We have now identified 19-hydroxy-Δ^4-androstene-3,17-dione-C^{14} after the incubation of Δ^4-androstene-3,17-dione-4-C^{14} with a human placental cell-free preparation.

MATERIAL AND METHODS

A preparation was obtained from human placentas by a method similar to that of Ryan (2, 17). Human term placentas were obtained immediately after delivery. After removal of the connective tissue and fetal membrane, the placental tissue was washed with distilled water, dried between two sheets of absorbent paper, weighed and homogenized in a Waring blendor for one minute in solution of 1.15% KCl. These procedures were carried out at 4° C. One volume of solution to three parts of tissue by weight was used. The homogenate was then subjected to differential centrifugation and the fraction obtained between 5,000 g. and 105,000 g. was resuspended in phosphate buffer 0.1 M (pH 7).

For each incubation one hundred μg. of C^{14}-labelled steroid dissolved in 0.1 ml. of propylene glycol was added to 2 ml. of microsomal suspension and the following substances added: TPN (triphosphopyridine nucleotide), 4.6 μ moles, glucose-6-phosphate, 8.5 μ moles, and glucose-6-phosphate dehydrogenase, 1 unit. The final volume was 3 ml. The incubations were carried out in a Dubnoff shaking incubator at $37° \pm 1°$ for one hour, with air as the gas phase.

Received November 12, 1959.

[1] Supported in part by U. S. Public Health Service Grants A-2672, CRTY-5001 and CY-757(C6).

[2] Fellow of French Government (Cultural Relations).

Extraction of the incubation mixture was accomplished with chloroform (2, 17). In some experiments a silica gel column (the Davidson Chemical Corporation, mesh size 30 to 200, 50 parts silica gel, one part dry residue) was used to remove the fat. The column was prepared in benzene and the residue applied to the column in benzene. The first two benzene fractions were discarded, then the column eluted with ethyl-acetate, chloroform, acetone and methanol. In other experiments the dried residue was partitioned between 90% methanol and ligroin.

The pooled methanol from the partition or the solvents from the column elution were evaporated and the residues dissolved in 20 ml. of toluene. The toluene was extracted four times with 20 ml. of 1 N NaOH and twice with 5 ml. of water. The combined aqueous phases were adjusted to pH 8.7 and extracted three times with diethyl ether (4). The ether was evaporated and the residues subjected to an eight transfer countercurrent distribution in toluene—1 N NaOH according to the procedure described by Baggett *et al.* (5). This procedure was carried out in centrifuge tubes with the upper phase transferred.

For counting, suitable aliquots of samples were poured in vials (low potassium erylite) and evaporated to dryness. Fifteen ml. of toluene (special purity for scintillation work, Matheson, Coleman and Bell) containing 4 g. diphenyl-oxazole and 100 mg. 1,4-di(2-[5-phenyloxazolyl])-benzene per liter was added and counted in a Tri-Carb Liquid scintillation spectrometer, model 314 (Packard Instrument Company, Inc.). All counts reported are corrected for background.

The radioactivity on paper chromatograms was recorded by means of a Chromatogram Scanner R.S.C. 5 (Atomic Accessories, Inc.).

Paper chromatographic separations were carried out at 25° ± 1° C according to the general method outlined by Savard (6), Zaffaroni *et al.* (7) and Bush (8) using washed Whatman No. 1 paper. Four solvent systems were employed: ligroin saturated with propylene glycol; toluene saturated with propylene glycol (paper strips impregnated with methanol-propylene glycol (1:1); chloroform saturated with formamide (paper strips impregnated with formamide methanol 1:1); and 66% ligroin 34% benzene (mobile phase) and 80% methanol 20% water (stationary phase) for the Bush System, B3. To detect the different compounds on the paper chromatograms, Turnbull's blue reagent (mixture of equal parts of aqueous ferric chloride 1% and ethanolic potassium ferricyanide 1%) was used for the phenolic substances, and ultraviolet absorption, Zimmermann reaction adapted for paper, and isonicotinic acid hydrazide (strong reagent) (9) were used to locate other compounds.

EXPERIMENTAL

The residue of an extract obtained after incubation of 100 μg. of Δ^4-androstene-3,17-dione-4-C^{14} (0.753 μc.)[3] with a placental microsome containing 13 mg. of nitrogen was applied on chromatographic paper (1×48 cm.) and developed in ligroin for 12 hours, then in toluene for 3 hours. The radioactive compounds were detected by radioautography. In addition to estrone and estradiol-17β, four major radioactive zones were found. Zone I remained at the origin, absorbed ultraviolet light, gave a brown color with the Zimmermann reagent and a yellow color and fluorescence with isonicotinic acid hydrazide under ultraviolet light at 365 mμ. No further identification of this material was done.

Zone II absorbed in the ultraviolet region, gave a purple color with the

[3] Obtained from New England Nuclear Corp., Boston, Massachusetts.

UNIVERSITY OF MICHIGAN LIBRARIES

Zimmermann reagent and a yellow color and fluorescence with isonicotinic acid hydrazide. Its mobility, relatively low, was between that of estradiol-17β and estrone and was identical to that of an authentic sample of 19-hydroxy-Δ^4-androstene-3,17-dione (18, 19) run on the same chromatogram. Zone III absorbed ultraviolet light, gave a brown color with the Zimmermann reagent, yellow color and fluorescence with isonicotinic acid hydrazide. Its migration rate corresponded to that of testosterone. Zone IV absorbed ultraviolet light, gave a purple color with the Zimmermann reagent, a yellow color and fluorescence with isonicotinic acid hydrazide. Its migration rate corresponded to that of Δ^4-androstene-3,17-dione.

Zone II was eluted and rechromatographed in toluene for 23 hours and its migration rate was again equal to that of 19-hydroxy-Δ^4-androstene-3,17-dione. One mg. of unlabelled 19-hydroxy-Δ^4-androstene-3,17-dione was added to Zone II and the material chromatographed in four different systems: ligroin-propylene glycol (48 hours); toluene-propylene glycol (12 hours); chloroform-formamide (3 hours); and Bush System (B3) (3 hours). In all four systems the peak of radioactivity corresponded exactly to the position of an authentic sample of 19-hydroxy-Δ^4-androstene-3,17-dione. The zones from the four chromatograms corresponding to the 19-hydroxy steroid were combined and contained a total of 6,700 c.p.m., an additional 10 mg. of 19-hydroxy-Δ^4-androstene-3,17-dione was added and the mixture crystallized from acetone-ether to constant specific activity. The specific activities for four successive recrystallizations were 587, 661, 651, and 650 c./min./mg. respectively.

The 19-acetoxy derivative of 19-hydroxy-Δ^4-androstene-3,17-dione-C^{14} was prepared, applied to chromatographic paper (3.5 cm.$\times$4.8 cm.) and developed in the ligroin-propylene glycol system for 40 hours. The radioactivity was associated with the compound which on the basis of mobility and infrared spectra had the properties of 19-hydroxy-Δ^4-androstene-3,17-dione acetate. Attempts to crystallize this material failed.

If the radioactive material from Zone II was indeed 19-hydroxy-Δ^4-androstene-3,17-dione-C^{14}, it should, on incubation with the placental preparation, be transformed to radioactive estrone. To test this possibility, 1.1 mg. of the compound from the fourth recrystallization was incubated with the placental preparation. After extraction, 3 mg. of carrier estrone was added. Fat from the residues was removed by partition between ligroin and 90% methanol, followed by silica gel column chromatography. The phenolic fraction obtained after partition between toluene and 1 N NaOH was purified by an eight transfer countercurrent distribution (5). The pooled phenolic fraction was applied on 5$\times$48 cm. chromatographic paper and run in Bush 3 System for 3 hours. The zone corresponding to estrone was eluted and after crystallization from acetone-methanol yielded material having a specific activity of 131 c./mg./min. Three recrystallizations yielded specific activities of 131, 149, and 127 respectively.

DISCUSSION AND CONCLUSION

The conversion of C_{19} steroids to estrogens has been amply demonstrated (5, 11, 12) and the fact that the C_{19}-19-hydroxy compound is an intermediate in estrogen biosynthesis strongly indicated (1, 2, 3). It has now been demonstrated for the first time that in an incubation using a human placental preparation with Δ^4-androstene-3,17-dione as the substrate that in addition to a good yield of estrone, 19-hydroxy-Δ^4-androstene-3,17-dione could be demonstrated. On incubation of Δ^4-androstene-3,17-dione with the placental system, Ryan (2) observed a product which appeared to have the properties of 19-hydroxy-Δ^4-androstene-3,17-dione on the basis of mobility in a paper chromatographic system, Zimmermann positive test, and ultraviolet absorption.

The presence of the 19-hydroxylase in placenta tissue has been demonstrated. Previous direct demonstrations for this enzyme system have implicated only the adrenal gland. 19-Hydroxy-Δ^4-androstene-3,17-dione (13) and 19-hydroxy-11-desoxycorticosterone (10, 14, 15, 16) were isolated from adrenal incubations using the corresponding steroids without a 19-hydroxyl group and progesterone perfused through bovine adrenals yielded 19-hydroxy-11-desoxycortisol and 19-hydroxydesoxycorticosterone (16). Indirect evidence for 19-hydroxylase in testis, ovary and placenta was based on the overall conversion of C_{19} compounds to estrogens.

REFERENCES

1. Gual, C., J. E. Longchampt and R. I. Dorfman: In preparation.
2. Ryan, K. J.: *J. Biol. Chem.* **234:** 268. 1959.
3. Meyer, A. S.: *Biochim. et Biophys. Acta* **17:** 441. 1955.
4. Engel, L. L., W. R. Slaunwhite Jr., P. Carter and I. T. Nathanson: *J. Biol. Chem.* **185:** 255. 1950.
5. Baggett, B., L. L. Engel, K. Savard and R. I. Dorfman: *J. Biol. Chem.* **221:** 931. 1956.
6. Savard, K.: *Recent Progress in Hormone Research* **9:** 185. 1954.
7. Zaffaroni, A.: *Recent Progress in Hormone Research* **8:** 51. 1953.
8. Bush, I. E.: *Biochem. J.* **50:** 370. 1952.
9. Smith, L. L. and T. Foell: *Anal. Chem.* **31:** 102. 1959.
10. Zaffaroni, A., V. Troncoso and M. Garcia: *Chemistry and Industry.* 534. 1955.
11. Heard, R. D. H., P. H. Jellinck and V. J. O'Donnell: *Endocrinology* **57:** 200. 1955.
12. West, C. D., B. L. Damast, S. D. Sarro and O. H. Pearson: *J. Biol. Chem.* **218:** 409. 1956.
13. Meyer, A. S.: *Experientia* **11:** 99. 1955.
14. Hayano, M. and R. I. Dorfman: *Arch. Biochem. & Biophys.* **55:** 289. 1955.
15. Kahnt, F. W., R. Neher and A. Wettstein: *Helv. chim. Acta* **38:** 1237. 1955.
16. Levy, H. and S. Kushinsky: *Arch. Biochem. & Biophys.* **55:** 299. 1955.
17. Ryan, K. J.: *Biochim. et Biophys. Acta* **27:** 658. 1958.
18. Ehrenstein, M. and M. Dünnenberger: *J. Org. Chem.* **21:** 774. 1956.
19. Ehrenstein, M. and K. Otto: *J. Org. Chem.* **24:** 1959.

UNIVERSITY OF MICHIGAN LIBRARIES

STUDIES ON THE EFFECT OF OMEGA-METHYL-PANTOTHENIC ACID ON CORTICOSTERONE SECRETION IN THE RAT

A. DAVID GOODMAN

Endocrinology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland

ABSTRACT

Adrenal venous blood was collected from pantothenic acid deficient rats, and the rate of secretion of corticosterone, the predominant corticosteroid in the rat, was determined. A decrease in corticosterone output was demonstrated, which occurred before the development of the hemorrhagic adrenal necrosis typical of pantothenic acid deficiency. Omega-methylpantothenic acid also produced a significant reduction of corticosterone secretion, which was reversible over a 24 hour period by pantothenic acid. Chronic administration of ACTH blocked the inhibitory effect of omega-methylpantothenic acid on corticosterone secretion. Omega-methylpantothenic acid did not produce hemorrhagic adrenal necrosis, even in rats in which a hemorrhagic tendency had been produced by treatment with sodium warfarin.

The potentialities of omega-methylpantothenic acid as a pharmacologic agent for the suppression or destruction of the adrenal cortex are discussed.

HEMORRHAGIC necrosis of the fasciculata and reticularis of the adrenal cortex is known to occur in the pantothenic acid deficient rat (1–3). There is indirect evidence of relative insufficiency of the adrenal cortex in this deficiency state (4, 5) and direct studies of corticosteroid secretion appear to confirm this. It has been demonstrated that the adrenals of pantothenic acid deficient rats, when incubated *in vitro*, secrete a reduced amount of Δ4-3-ketosteroids (6). Longwell *et al.* (7) found that in rats fed a pantothenic acid deficient diet supplemented with omega-methylpantothenic acid, there was reduced corticosterone output as measured by adrenal vein cannulation. Eisenstein and Wenneker (8) placed guinea pigs on a similar regimen and demonstrated failure of these animals to respond to ACTH with a normal rise in urinary 17-hydroxycorticosteroid excretion.

Omega-methylpantothenic acid, an analogue of pantothenic acid, causes retardation of growth in the mouse (9) and in the rat (10, 11), an effect which is both preventable and reversible by the administration of sufficient amounts of pantothenic acid. The potentialities of omega-methylpantothenic acid as an inhibitor of adrenal function have yet to be investigated. The impairment of adrenal function found in animals fed a pantothenic acid deficient diet plus this antagonist, as compared with an-

Received August 17, 1959.

nals on a diet containing pantothenic acid (7, 8), may have been due to the antothenic acid deficiency per se, with the antagonist contributing nothing to this impairment.

Moreover, one cannot assume a priori that because omega-methylpantothenic acid is an antagonist of pantothenic acid with regard to growth that it will also interfere with the role of the vitamin in adrenal function; for an analogue of a given metabolite may interfere with some of the biologic functions of the metabolite without affecting others, a phenomenon of which many examples can be cited (12–14). A particularly relevant example is the pantothenic acid antagonist pantoylaminoethanethiol, which causes a retardation of growth in the rat that is preventable by the administration of the vitamin, yet does not produce any of the histologic changes in the adrenal that are characteristic of pantothenic acid deficiency (15).

It was the primary purpose of this investigation to observe the effects of omega-methylpantothenic acid on rat adrenal function and histology and compare them with the effects of pantothenic acid deficiency. Secondly, the effect of combined administration of omega-methylpantothenic acid and ACTH was studied. Third, the possibility was explored that the production of a hemorrhagic tendency with sodium warfarin would intensify any hemorrhagic adrenal damage that might be induced by the pantothenic acid antagonist.

MATERIALS AND METHODS

Male Holtzman rats, housed individually in screen-bottom cages, were used in all experiments.

The basic pantothenic acid deficient diet employed (to which calcium pantothenate and calcium omega-methylpantothenate were added where specified) contained the following components per 100 gm. diet: sucrose, 68 gm.; "vitamin free" casein (Nutritional Biochemical Corp.), 18 gm.; vegetable oil, 10 gm.; USP salt mixture No. 2, 4 gm.; vitamin A, 2,000 units; vitamin D, 220 units; α-tocopherol, 11.0 mg.; inositol, 11.0 mg.; choline chloride, 165.0 mg.; menadione, 5.0 mg.; p-amino benzoic acid, 11.0 mg.; niacin, 10.0 mg.; riboflavin, 2.2 mg.; pyridoxine hydrochloride, 2.2 mg.; thiamine hydrochloride, 2.2 mg.; biotin, 44 μg.; folic acid 200 μg.; and vitamin B_{12}, 3.0 μg.

The omega-methylpantothenic acid was administered as calcium omega-methylpantothenate.[1]

[1] Obtained from California Foundation for Biochemical Research, Los Angeles, California (Lot 090960).

Because not all lots of this analogue are active as pantothenic acid antagonists (16), the potency of the lot used in these experiments was determined by both bacteriologic and rat growth assay. The inhibition ratio for Lactobacillus arabinosus 8014 for 50% suppression of growth as measured turbidimetrically at 24 hours was 1,000:1 for pantothenic acid levels of 0.1 μg. amd 0.2 μg. per tube. For 50–60 gm. rats on ad lib diet containing 0.3 mg. pantothenic acid per 100 gm., the ratio of omega-methylpantothenic acid to pantothenic acid necessary to cause significant retardation of growth over a 7 day period was 450:1. The inhibition of both bacterial and rat growth was preventable by the addition of more pantothenic acid.

UNIVERSITY OF MICHIGAN LIBRARIES

In all of the experiments to be described, the control animals were daily individually pair-fed to the experimental animals (deficient or antagonist-treated) as soon as the latter developed anorexia.

In the studies of pantothenic acid deficiency, 30–40 gm. rats were fed a pantothenic acid deficient diet and control animals were pair-fed the same diet to which was added 10 mg. of calcium pantothenate per 100 gm. About the fiftieth day, when the deficient animals were manifesting considerable growth retardation and other gross symptoms, adrenal vein cannulation was performed.

In one study on the effect of omega-methylpantothenic acid, rats weighing 50–60 gm. received a diet containing 0.3 mg. pantothenic acid and 0.5 gm. omega-methylpantothenic acid per 100 gm., while controls were pair-fed the same diet devoid of the antagonist. Adrenal vein blood was collected on the 18th day.

Another study of the effect of omega-methylpantothenic acid and of its reversibility by pantothenic acid was designed as follows: 50–60 gm. rats were placed on a pantothenic acid deficient diet containing 0.75 gm. antagonist per 100 gm. and control animals were pair-fed the same diet without the antagonist. About the 11th day, adrenal vein cannulation was performed on half of the antagonist-treated animals and their pair-fed controls. On that day the other animals received an injection of 65 mg. of calcium pantothenate in 1.0 cc. distilled water subcutaneously and adrenal vein blood was collected 24 hours later. (It was noted that considerable necrosis occurred at the site of the injections but this was of equal extent in antagonist-treated and control animals.)

In the studies of the combined effects of ACTH and omega-methylpantothenic acid, the diets and the duration of the experiments were the same as in the preceding study. ACTH was given to half of the antagonist-treated and untreated rats according to one of the following two regimens: a) ACTH (Armour) 2 units in 0.1 cc. saline subcutaneously 4 times daily starting 1 day before the start of the antagonist, plus 2 units in saline subcutaneously 20 minutes before cannulation. b) ACTH 4 units in 0.1 cc 5% beeswax in peanut oil suspension subcutaneously daily starting 5 days before cannulation, plus 4 units in saline subcutaneously 20 minutes before cannulation.

In the study of the effect of combined treatment with omega-methylpantothenic acid and an anti-coagulant drug, the diets and the duration of the experiment were the same as in the preceding study, but both antagonist-treated and untreated rats were given .02 mg. of sodium warfarin intraperitoneally daily for 4–5 days before sacrifice. Production of a hemorrhagic tendency by the last day of warfarin administration was demonstrated by the development of large hematomas at the sites of subcutaneous saline injections (0.5 cc). The hemorrhagic tendency was reversed by the administration of 10 mg. of vitamin K_1 oxide and 2 mg. of menadione intraperitoneally 16–24 hours before sacrifice. Antagonist-treated rats not receiving warfarin were run simultaneously for comparison.

Adrenal vein blood was collected for 12.5 minutes under ether anesthesia without previous fasting. The cannulation procedure employed was similar to that of Longwell *et al.* (7) except that the major tributaries of the left renal vein and of the left adrenal vein were ligated. Following the collection the animal was sacrificed, the right adrenal fixed for histologic examination and the left adrenal weighed.

The adrenal vein plasma concentration of corticosterone, the predominant corticosteroid in the rat, was then measured by a slight modification of the method of Peterson (17, 18). This involved extraction of 0.05 cc. plasma with 7 cc. of methylene chloride; extraction of a 4 cc. aliquot of the methylene chloride with 1.5 cc. of 37:63 ethanol-sulfuric reagent; and measurement of the fluorescence of the latter 2–4 hours later in a Farrand fluorometer (the following filter combinations being used: primary filter, 436 μ and No. 5513 Corning glass filter; secondary filters 521 μ interference and No. 3389 Corning glass filter). Corticosterone standards containing 0.2 and 0.4 μg. per cc. were

TABLE 1. ADRENAL CORTICOSTERONE SECRETION IN PANTOTHENIC ACID DEFICIENCY

Description	No. rats	Weight (gm.)	Left adrenal wt. (mg.)	Left adrenal plasma flow (ml./min.)	Corticosterone output (μg./min./adrenal)**
A. Deficient	14	84	17.2±10.0	0.023	0.18±.10
B. Control*	14	102	13.7± 1.5	0.023	0.34±.09†

* Pair-fed the deficient diet plus 10 mg. pantothenic acid per 100 gm. diet. All animals on diets for an average of 50 days.
** ±Standard error.
† Difference from group A statistically significant, P = <0.01.

read with the unknowns and all determinations were done in duplicate. This method has been demonstrated by Peterson, by means of an isotope dilution technique, to be specific for corticosterone in rat adrenal vein plasma and to give a recovery of about 95% (18).

The right adrenal glands, after fixation in Baker's formol solution followed by Bouin's solution, were embedded in paraffin and semi-serial sections of one-half of each gland were made every 150 micra which were stained with hematoxylin-eosin.

RESULTS

Effects of Pantothenic Acid Deficiency

Rats in the pantothenic acid deficiency state secreted 47% less adrenal corticosterone than did pair-fed control rats, as seen in Table 1. Adrenal necrosis was seen microscopically in only 4 of the 14 deficient rats. If one considers only those rats in which adrenal necrosis had not yet developed (Table 2) corticosterone secretion was still 42% less than in the controls.

The adrenals of the pantothenic acid deficient rats were characteristically greyish-purple in color as compared to the light pink seen in the controls. In those deficient animals in which adrenal necrosis did not occur, histology of the adrenal fasciculata and reticularis differed from that in the control animals primarily in that there was almost complete absence of the normal vacuolization of the cytoplasm.

Effects of Omega-Methylpantothenic Acid

Rats placed on a diet containing 0.0003% pantothenic acid and 0.5% omega-methylpantothenic acid for 18 days secreted 39% less corticosterone than did rats pair-fed the same diet devoid of antagonist (Table 3).

TABLE 2. ADRENAL CORTICOSTERONE SECRETION IN PANTOTHENIC ACID DEFICIENCY DURING THE PRE-NECROSIS PERIOD

Description	No. rats	Weight (gm.)	Left adrenal wt. (mg.)	Left adrenal plasma flow (ml./min.)	Corticosterone output (μg./min./adrenal)
A. Deficient	10	84	10.8±3.1	0.025	0.21±.09
B. Control	10	99	12.0±1.3	0.026	0.36±.08†

† Difference from group A statistically significant, P = <0.01.

UNIVERSITY OF MICHIGAN LIBRARIES

TABLE 3. ADRENAL CORTICOSTERONE SECRETION IN RATS TREATED WITH OMEGA-METHYLPANTOTHENIC ACID

Description*	No. rats	Weight (gm.)	Left adrenal wt. (mg.)	Left adrenal plasma flow (ml./min.)	Corticosterone output (μg./min./adrenal)
A. Antagonist (500:0.3)**	4	66	11.6 ± 1.7	0.019	0.17 ± .02
B. Untreated (0:0.3)	4	79	13.0 ± 1.0	0.017	0.28 ± .03†

* Figures in parentheses indicate ratio of omega-methylpantothenic acid in mgm. per 100 gm. diet to pantothenic acid in mgm. per 100 gm. Animals were on diet for 18 days.
** Average daily intake of omega-methylpanothenic acid was 400 mg. per kg. body weight.
† Difference from group A statistically significant, P = <0.01.

Similarly, rats receiving a pantothenic acid deficient diet containing 0.75% omega-methylpantothenic acid for 11 days secreted 46% less corticosterone than did rats pair-fed the same diet without antagonist (Table 4, Groups A and B). In the same experiment, however, those antagonist-treated animals which received pantothenic acid injections on the 11th day, with cannulation of the adrenal vein 24 hours later, did not differ significantly in rate of corticosterone secretion from their controls (Groups C and D). The difference between the corticosterone output of the antagonist-treated rats which received a "reversing dose" of pantothenic acid and of those which did not is statistically significant.

In the experiment just described the adrenals of all of the antagonist-treated animals which had not received pantothenic acid were greyish-purple in color. No adrenal hemorrhage or necrosis was observed microscopically, but there was a decrease in cytoplasmic vacuolization in most of these animals. Normal color and normal vacuolization were noted in antagonist-treated animals which had received a "reversing dose" of pantothenic acid.

TABLE 4. EFFECT OF ADMINISTRATION OF PANTOTHENIC ACID 24 HOURS EARLIER ON ADRENAL-INHIBITORY EFFECT OF OMEGA-METHYLPANTOTHENIC ACID

Description*	No. rats	Weight (mg.)	Left adrenal weight (mg.)	Left adrenal plasma flow (ml./min.)	Corticosterone output (μg./min./adrenal)
A. Antagonist (750:0)**	6	60	9.3 ± 1.2	0.026	0.15 ± .03
B. Untreated (0:0)	6	69	11.1 ± 2.2	0.033	0.28 ± .04†
C. Antagonist (750:0)** plus pantothenic acid	7	63	10.7 ± 0.7	0.028	0.24 ± .05†
D. Untreated (0:0) plus pantothenic acid	7	67	12.0 ± 1.4	0.028	0.27 ± .04

* Figures in parentheses indicate ratio of omega-methylpantothenic acid in mg. per 100 gm. diet to pantothenic acid in mg. per 100 gm. Groups A and B were on diet for 11 days, and groups C and D for 12 days.
** Average daily intake of omega-methylpantothenic acid was 500 mg. per kg. body weight.
† Difference from group A statistically significant, P = <0.01.

Effects of ACTH plus Omega-Methylpantothenic Acid

Antagonist-treated rats and control rats which received massive doses of ACTH for 5–13 days before cannulation, had similar corticosterone secretion rates (Table 5, Groups A and B). In contrast, the previously demonstrated difference in corticosterone output between antagonist-treated and control rats was evident in those groups in the same experiment which did not receive ACTH (Groups C and D). No evidence of adrenal necrosis was found in this series on gross examination.

Effects of Sodium Warfarin plus Omega-Methylpantothenic Acid

Induction of a hemorrhagic tendency with sodium warfarin in 6 rats treated with omega-methylpantothenic acid for 13 days failed to result in gross or histologic changes not seen in 4 rats treated with the antagonist alone. The previously described adrenal cytoplasmic changes were evident, but no hemorrhagic damage was observed.

DISCUSSION

The results of the first part of this study confirm the findings of Longwell, *et al.* (7) that in pantothenic acid deficiency in the rat there is a reduced adrenal secretion of corticosterone, under the conditions described. In addition it was demonstrated that the decrease in corticosteroid synthesis preceded the development of adrenal necrosis.

The studies of omega-methylpantothenic acid clearly indicate that this antagonist per se impairs adrenal biosynthesis of corticosterone. The ready reversibility by pantothenic acid of this impairment indicates that it is due specifically to interference by the antagonist with pantothenic acid metabolism. The importance of pantothenic acid in adrenal metabo-

TABLE 5. EFFECT OF COMBINED TREATMENT WITH ACTH AND OMEGA-METHYLPANTOTHENIC ACID ON CORTICOSTERONE SECRETION

Description*	No. rats	Weight (gm.)	Left adrenal weight (mg.)	Left adrenal plasma flow (ml./min.)	Corticosterone output (μg./min./adrenal)
A. Antaongist (750:0)+ACTH**	6	53	21.9±4.7‡	0.029	0.37±.11†
B. Untreated (0:0) +ACTH**	6	56	29.0±5.8	0.032	0.36±.08
C. Antagonist (750:0)	5	62	11.5±1.7	0.021	0.12±.06
D. Untreated (0:0)	5	67	12.3±1.0	0.031	0.30±.20†

* Figures in parentheses indicate ratios of omega-methylpantothenic acid in mg. per 100 gm. diet to pantothenic acid in mg. per 100 gm. Animals were on diet for 12–13 days.

** Three rats of group A and 3 of Group B received 8 units of ACTH (Armour) in saline daily from 1 day before the antagonist was begun until cannulation, plus 2 units in saline 20 minutes before cannulation. The remainder received 4 units of ACTH in beeswax-peanut oil suspension daily for 5 days before cannulation plus 4 units in saline 20 minutes before cannulation.

† Difference from group C statistically significant, P = <0.01.

‡ Difference from group B not statistically significant, P = >0.50.

lism is thought to be due to the fact that it constitutes a moiety of coenzyme A (19) which is known to be a factor in the synthesis of steroids from acetate (20). Presumably omega-methylpantothenic acid interferes with adrenal steroid synthesis by interfering with either the formation or function of coenzyme A.

The failure of omega-methylpantothenic acid to cause adrenal necrosis may have been due to the brief period of administration. Even if complete blockage of the synthesis of an indispensable compound were effected by the antagonist, a minimum amount of time would have to elapse before existing stores of the essential compound were depleted below the level required for cell viability. If this is true, an agent which could increase the turnover rate of the essential substance might speed the development of adrenal necrosis in the antagonist-treated animal. These considerations led to the study of the influence of large doses of ACTH on the effectiveness of the antagonist. It appeared to be a particularly promising approach in view of the finding of Schultz *et al.* that ACTH administration intensified the adrenal necrosis of pantothenic acid deficiency (3).

The antagonist-treated animals which received ACTH did not develop adrenal necrosis. That they put out significantly more corticosterone than did the antagonist-treated animals which did not receive ACTH, could be accounted for by one of the following possibilities: a) The ACTH preparation may have contained sufficient pantothenic acid to block the effect of the antagonist; b) Under intense ACTH stimulation synthesis of coenzyme A or some other key metabolite may proceed by a metabolic route not blocked by omega-methylpantothenic acid; c) Partial insufficiency of endogenous ACTH production may contribute to the decreased corticosterone secretion seen in the antagonist-treated animals and this would would be compensated for by the administered ACTH. This last alternative seems least likely in view of the findings of normal blood and pituitary ACTH in pantothenic acid deficient rats (21).

Production of a hemorrhagic tendency with warfarin seemed a possible means of precipitating hemorrhagic adrenal necrosis in antagonist-treated rats, particularly in view of the fact that spontaneous hemorrhagic destruction of the adrenals occasionally occurs in patients receiving anticoagulant drugs (22). Failure of warfarin to affect the adrenal in this study may have been due to the fact that the omega-methylpantothenic acid had yet to produce any early adrenal hemorrhagic changes.

The demonstration that omega-methylpantothenic acid has an inhibitory effect on adrenal cortical function suggests that it might prove useful as a pharmacologic agent for the suppression or destruction of normal or neoplastic adrenal tissue. Search for an agent that would increase the vulnerability of the adrenal to this antagonist might consititute a particularly rewarding approach.

Acknowledgment

The encouragement and advice of Drs. Roy Hertz and William W. Tullner are gratefully acknowledged. The author also wishes to thank Mr. Howard Erwin for having prepared the histologic sections.

REFERENCES

1. Daft, F. S., W. H. Sebrell, S. H. Babcock, Jr. and T. H. Jukes: *Public Health Reports* **55:** 1333. 1940.
2. Deane, H. W. and J. M. McKibbin: *Endocrinology* **38:** 385. 1946.
3. Schultz, R. B., R. W. Winters and W. A. Krehl: *Endocrinology* **51:** 336. 1952.
4. Winters, R. W., R. B. Schultz and W. A. Krehl: *Endocrinology* **50:** 377. 1952.
5. Winters, R. W., R. B. Schultz and W. A. Krehl: *Endocrinology* **50:** 388. 1952.
6. Eisenstein, A. B.: *Endocrinology* **60:** 298. 1957.
7. Longwell, B. B., A. E. Reif and E. Hansbury: *Endocrinology* **62:** 565. 1958.
8. Eisenstein, A. B. and A. S. Wenneker: *Proc. Soc. Exper. Biol. & Med.* **97:** 844. 1958.
9. Drell, W. and M. S. Dunn: *Arch. Biochem. & Biophys.* **33:** 110. 1951.
10. Drell, W.: Dissertation, U. of California, Los Angeles, 1949.
11. Goodman, A. D.: Unpublished data.
12. Woolley, D. W.: A Study of Antimetabolites. New York, John Wiley and Sons, Inc., 1952, p. 79.
13. Eusebi, A. J. and L. R. Cerecedo: *Science* **110:** 162. 1949.
14. Sangster, W., M. I. Grossman and A. C. Ivy: *Gastroenterology* **6:** 436. 1946.
15. Boxer, G. E., C. E. Shonk, H. C. Stoerck, T. C. Bielinski and T. V. Budzilovich: *J. Biol. Chem.* **217:** 541. 1955.
16. Hodges, R. E.: Personal communication.
17. Peterson, R. E.: Personal communication.
18. Peterson, R. E.: *J. Biol. Chem.* **225:** 25. 1957.
19. Gregory, J. D., G. D. Novelli and F. Lipmann: *J. Am. Chem. Soc.* **74:** 854. 1952.
20. Klein, H. P. and F. Lipmann: *J. Biol. Chem.* **203:** 101. 1953.
21. Butler, L. C. and A. F. Morgan: *Endocrinology* **56:** 322. 1955.
22. Chokas, W. V.: *Am. J. Med.* **24:** 454. 1958.

COMPENSATORY ADRENAL HYPERTROPHY AS A POSSIBLE CAUSE OF METACORTICOID HYPERTENSION[1]

T. OMAE AND G. M. C. MASSON

Research Division, Cleveland Clinic Foundation and Frank E. Bunts Educational Institute, Cleveland, Ohio

ABSTRACT

Desoxycorticosterone acetate (DCA), cortisone or cortisol elicited hypertensilon which, after discontinuation of treatment, persisted only in the DCA-treated group. This self-sustained hypertension is not dependent upon adrenal hypertrophy subsequent to compensatory adrenal atrophy, nor on the presence of renal lesions.

DCA and cortisol, but not cortisone, caused nephrosclerosis; similar lesions accompanied by massive myocardial hemorrhages and necroses were obtained with ACTH but only after cortisone pretreatment.

SINCE adrenal regeneration causes hypertension (1), it has been postulated (2) that the adrenal hypertrophy subsequent to steroid-induced atrophy may be of pathogenic significance in the establishment of metacorticoid hypertension (3). The present experiments, in which we used desoxycorticosterone (DCA), cortisone and cortisol, were devised to test this hypothesis. The role of adrenal hypertrophy was further investigated by treatment with ACTH following discontinuation of steroid therapy.

METHODS

Female Sprague-Dawley rats weighing 90 to 111 gm. were uninephrectomized and uniadrenalectomized; the purpose of the latter was to accentuate contralateral adrenal hypertrophy. DCA, cortisone and cortisol were tested respectively in 3 experimental series. Each series consisted of 4 groups of 8 to 12 rats which received the same steroid treatment during the first 15 days. Then differences in treatment were as follows: in the first group, treatment was continued until the 30th day; the second group was kept without any further treatment; the third group was killed on the 16th day, and the fourth group received ACTH during the following 15 days. Thus, all rats except those of the third group were sacrificed on the 30th day, including those of an untreated control group. Steroids were administered as acetates in saline suspensions at the daily dosage of 1 mg. (DCA), 2.5 mg. (cortisone), and 2 mg. (cortisol). The daily dose of ACTH (Depo-ACTH Upjohn) was 8 units. Drinking fluid consisted of 1% NaCl solution and food was Purina fox chow. Blood pressure was measured twice weekly by tail plethysmography (4). At the end of the experiment tissues were fixed in Susa fluid, weighed and prepared for histological examination.

Received August 24, 1959.

[1] Supported by a grant (H-96) from the National Heart Institute.

TABLE 1. PHYSIOLOGIC AND MORPHOLOGIC EFFECTS ASSOCIATED WITH ADRENAL RECOVERY FOLLOWING DISCONTINUATION OF STEROID TREATMENT*

Groups	No. of rats	Treatment		Final body weight (gm.)	Final blood pressure (mm.Hg.)	Organ weights in per cent of body weight		
		0 to 15th day	15th to 30th day			Kidney (gm.)	Heart (gm.)	Adrenal (mg.)
I	7	DCA	DCA	139± 9.9	220± 7.47	1.11±.064	.42±.028	18.5±1.58
II	7	DCA	0	144± 9.9	222± 5.16	1.00±.072	.45±.028	16.9±1.17
III	6	DCA		130± 7.06	166± 3.18	.93±.031	.38±.012	14.1± .88
IV	5	DCA	ACTH	133± 7.88	191±10.46	1.07±.126	.40±.033	27.5±2.82
V	10	Cortisone	Cortisone	128± 4.9	157± 6.5	.69±.010	.40±.014	6.4±0.69
VI	9	Cortisone	0	154± 5.4	131± 5.7	.60±.009	.32±.007	14.4±0.44
VII	10	Cortisone		94± 4.3	160± 6.3	.85±.014	.45±.013	6.9±0.44
VIII	5	Cortisone	ACTH	156±14.1	144± 9.0	.73±.037	.35±.019	20.6±1.55
IX	8	Cortisol	Cortisol	91± 5.1	161± 6.2	.94±.035	.45±.02	6.5± .6
X	11	Cortisol	0	123± 5.9	138± 6.7	.72±.02	.35±.007	5.6±1.6
XI	10	Cortisol		111± 3.3	153± 4.3	.72±.015	.34±.007	5.2± .23
XII	10	Cortisol	ACTH	123± 4.8	138± 5.2	.80±.024	.38±.014	17.1± .72
XIII	12	0	0	168± 6.1	131± 7.0	.71±.02	.32±.013	17.4± .55

* Values indicated are means ± standard error.

RESULTS

(Table 1, Fig. 1.) *Effects of DCA* (Groups I–IV). During DCA treatment the majority of rats rapidly became hypertensive so that on the 15th day, mean blood pressure averaged about 180 mm. Hg with a range of 145–210 mm. Hg. At that time, rats of Group III were sacrificed; 4 of them showed nephrosclerosis but no splanchnic arteritis. By comparison with the control Group XIII, kidneys and hearts were larger; the adrenals were only slightly atrophic.

Continuation of treatment (Group I) increased blood pressure further until it averaged 220 mm. Hg on the 30th day. Discontinuation of treatment (Group II) or substitution of ACTH for DCA (Group IV) resulted in about the same degree of hypertension and nephrosclerosis and increase in kidney and heart weights as in Group I. Adrenal weights were normal in Group I and II but almost twice normal after ACTH.

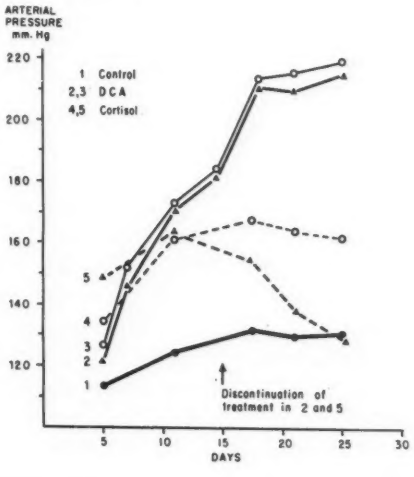
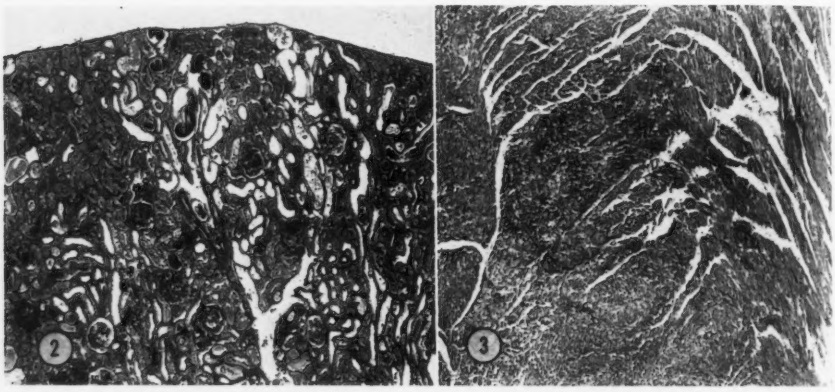


FIG. 1. Blood pressure curves of rats treated with DCA or cortisol showing effects of cessation of treatment.

Effects of Cortisone (Groups V–VIII). Cortisone caused an initial loss of body weight, followed by a slow increase; on discontinuation of cortisone or during ACTH treatment there was rapid recovery of normal growth. Three of 8 rats in the ACTH group died. Cortisone produced a rise in blood pressure which appeared earlier, but was not as great as when DCA was used; the maximum, reached on the 15th day, persisted throughout the experiment. It remitted completely on discontinuation of treatment but only partially with ACTH.

Heart weights in the cortisone treated groups were greater than in controls; they regressed to normal values on discontinuation of treatment. In the survivors of the ACTH group, mean heart weight was only slightly



FIGS. 2 and 3. Tissues from rats treated with ACTH following pretreatment with cortisol. Fig. 2. Kidney showing severe nephrosclerosis—×55. Fig. 3. Heart showing in the center a large necrotic and hemorrhagic area—×55.

increased; however, in the 3 that died, weights reached values up to 0.45 gm. per 100 gm. of body weight.

Adrenal weights that were markedly depressed by cortisone recovered partially following cessation of treatment; following ACTH, they were slightly larger than normal.

The only lesions were seen in the ACTH group where nephrosclerosis was present in 6 of 8 rats (Fig. 2), including the 3 that died. These animals at times showed pressures near 200 mm. Hg during ACTH treatment. On microscopic examination, the hearts of these animals showed massive hemorrhages, myocardial necrosis or hyalinization, and arteriolonecrosis (Fig. 3).

Effects of Cortisol (Groups IX–XII). The effects on body weight, blood pressure, kidney, heart and adrenal weights were essentially the same or similar to those found in the cortisone experiment except for a more sustained inhibitory effect on growth and adrenal weights. Adrenals did not

show any recovery on discontinuation of treatment, while blood pressure returned almost to normal; adrenal weight was restored by ACTH. In contrast with the animals treated with cortisone, microscopic examination of kidneys showed nephrosclerosis in 6 of 9 rats in Group IX, 2 of 11 rats in Group X and 4 of 10 rats in Group XII; there were none in Group XI killed on the 16th day. The lesions consisted of focal fibrinoid necrosis of the glomerular tuft, extensive exudate in sub-capsular glomerular space, tubular dilatation and casts. These lesions were no more severe in the ACTH treated group. Hearts of these animals showed slight lesions of arteriolonecrosis; there was no splanchnic arteritis.

DISCUSSION

These experiments demonstrate unequivocally that metacorticoid hypertension does not depend on compensatory adrenal hypertrophy. It was present only in DCA-treated rats, which did not show adrenal regeneration because DCA did not cause adrenal atrophy. It was absent in the cortisone or cortisol groups in which adrenals returned to control size (cortisone) or remained atrophic (cortisol). It was also not dependent on the presence of steroid-induced anatomical renal lesions; while post-DCA hypertension can take place in absence of definite renal lesions (5), no sustained hypertension was observed following withdrawal of cortisol treatment which caused macroscopic nephrosclerosis. Finally, since metacorticoid hypertension was observed after DCA but not after cortisone (6) or cortisol treatment, it can be assumed that DCA elicits permanent impairment of the mechanisms responsible for the maintenance of normal blood pressure. This is further evidence that mineralo-corticoid and gluco-corticoid hypertensions have different basic mechanisms.

The presence of nephrosclerosis in rats treated with cortisol and in those receiving ACTH following cortisone on one hand, and the absence of lesions with cortisone and lack of effect of ACTH after DCA or cortisol on the other, emphasize the elusive effects of these agents. Although there is agreement that cortisone and cortisol elicit hypertension, renal and vascular lesions are usually absent (7–11). ACTH has long been considered to be ineffective in causing experimental hypertension (12); however, severe hypertension (1, 13) and arteriosclerosis (14) have been reported recently. These observations demonstrate that the conditions required to obtain a definite cardiovascular effect with these hormonal agents still remain undefined.

REFERENCES

1. Skelton, F. R.: *Proc. Soc. Exper. Biol. and Med.* **90**: 342. 1955.
2. Masson, G. M. C. and A. C. Corcoran: *Arch. internat. pharmacodyn.* **114**: 322. 1958.
3. Sturtevant, F. M.: *Ann. Int. Med.* **49**: 1281. 1958.

4. Masson, G. M. C., F. del Greco, F. Olmsted and A. C. Corcoran: *J. Lab. and Clin. Med.* **41:** 729. 1953.
5. Friedman, S. M., C. L. Friedman and M. Nakashima: *J. Exper. Med.* **93:** 361. 1951.
6. Bergeron, G. A. and G. Bourbeau: *Laval med.* **20:** 3. 1955.
7. Knowlton, A. J., E. N. Loeb, H. C. Stoerk, J. P. White and J. F. Heffernan: *J. Exper. Med.* **96:** 187. 1952.
8. Masson, G. M. C., A. C. Corcoran, H. Dustan and I. H. Page: *Am. J. Med. Sc.* **224:** 175. 1952.
9. Friedman, S. M., C. L. Freidman and M. Nakashima: *Endocrinology* **53:** 633 1953.
10. Toussaint, C.: *Comp. rend. Soc. Biol.* **145:** 1427. 1951.
11. Herlant, M. and P. S. Timiras: *Acta Anatomica* **12:** 229. 1951.
12. Sayers, G.: *Physiol. Rev.* **30:** 241. 1950.
13. Selye, H. and P. Bois: *Endocrinology* **58:** 739. 1956.
14. Wexler, B. C. and R. F. Miller: *Science* **127:** 590. 1958.

RELATIVE SENSITIVITY OF DIFFERENT BIOLOGICAL RESPONSES TO THYROXINE[1]

EDWARD S. EVANS, LAWSON L. ROSENBERG
AND MIRIAM E. SIMPSON

The Institute of Experimental Biology and The Department of Anatomy, University of California, Berkeley, California

ABSTRACT

Different groups of female rats surgically thyroidectomized at 28 days of age were injected with doses of L-thyroxine varying from 0.25 to 5 μg. daily for 96 days. A dose of 0.5 μg. was required to a maintain body growth while doses greater than 1 and less than 5 were required to maintain metabolic rates at levels normal for the age. Red cell volume was normal at 1 and 5 μg. but at the lower doses there was no prevention of the anemia which follows thyroidectomy. Pituitary acidophils were normal at 1 μg. whereas the basophils required between 1 and 5 μg. for prevention of hyperplasia and hypertrophy. The ovaries were normal in structure and weight at 1 μg. The adrenals were normal only after the administration of the higher doses needed for maintenance of calorigenesis. These findings reemphasize the differential in biological effectiveness of thyroxine.

SCOW and Simpson, in their study of thyroidectomy in the newborn rat, noted that the metabolic rate of a small number of incompletely thyroidectomized rats was not markedly different from that of completely thyroidectomized rats, yet the animals grew at a rate nearly half that of normal (1). Recently, this observation has been confirmed on a large number of incompletely thyroidectomized rats by the present authors. If it is assumed that the subnormal growth of these animals reflected the partial maintenance of pituitary acidophils, and hence growth hormone production, by low levels of thyroxine, then the reports of Griesbach and Purves suggest an explanation (2, 3). They found that normal pituitary acidophil morphology could be maintained in thyroidectomized rats by one fifth the dose of thyroxine necessary to maintain pituitary basophils. In fact, 0.25 μg. of DL-thyroxine per 100 gm. of body weight for four weeks prevented degranulation of half the acidophil population with little or no effect on the characteristic enlargement and vacuolation of the basophils which occurs after thyroidectomy.

The present experiment was designed to reexamine, systematically, the efficacy in thyroidectomized rats of graded doses of l-thyroxine in main-

Received September 11, 1959.

[1] Supported in part by U. S. Public Health Service grants A-800(C2) and A-661 and by University of California research grant 762.

taining calorigenesis, body growth, and pituitary and target organ weight and morphology. Since it has been the experience of this and other laboratories that an intimate relationship exists between oxygen requirements and erythropoiesis (4, 5, 6), the degree of maintenance of red cell volume in these animals was also studied.

MATERIALS AND METHODS

Fifty female rats of the Long-Evans strain were surgically thyroidectomized at 28 days of age and divided into five equal groups of the same body weight. The rats of four groups were injected intraperitoneally, beginning the day of operation and continuing daily for 98 days, with sodium L-thyroxine in alkaline solution at the levels of 0.25, 0.5, 1 and 5 μg. One group of thyroidectomized and a group of six normal rats of the same age and weight were maintained as uninjected controls. All rats were fed an adequate diet of natural foodstuffs.[2] Room temperature was 76±1° F.

The rats were weighed every eighth day and body lengths were measured initially and terminally (tip of nose to tip of tail). Metabolic rates were determined immediately prior to thyroidectomy and every 16 days thereafter, with a closed circuit respiration apparatus, consisting of eight chambers, which was patterned after that described by Kleiber (7). In order to control variations incident to time of day or night in this nocturnal animal, the metabolic rates were measured simultaneously for representatives of all groups. The rats were fasted four hours, then allowed to adjust to the temperature of the apparatus (30±0.1° C) for one hour prior to oxygen consumption measurements. The liters of oxygen consumed per rat over a 2.5 hour experimental period were converted into calories per hour by the factor 4.80. The calories per hour per square meter of body surface were calculated by Benedict's formula for the rat:

$$\text{Area in m.}^2 = \frac{9 \times \sqrt[3]{(\text{Body Weight in gm.})^2}}{10{,}000}$$

The metabolic rate so described is the standard metabolic rate and not the basal metabolic rate as defined for human beings.

On day 97 the blood volume of each rat was determined by the Fe^{59} tagged red cell dilution method (8) which consists essentially in the measurement of the dilution of radioactivity of injected tagged red cells (0.03 μc. Fe^{59} in 0.1 cc. whole blood) in the circulation of recipient rats. The red cell volumes were calculated by multiplying the blood volume by the hematocrit which was determined simultaneously. The results are presented in terms of ml. of red blood cells per 100 gm. of final body weight. Hemoglobin concentrations were determined by the method of Turner (9) and the results are expressed as gm. of hemoglobin per 100 ml.

The animals were not exsanguinated as is routine in determination of blood volume; instead, a 1.25 ml. sample of blood was withdrawn from the external jugular vein. The rats were then immediately injected intraperitoneally with one microcurie of I^{131} for checking the completeness of thyroid removal. Twenty hours later the rats were sacrificed and the entire ventral neck region was removed,[3] placed in a vial, and the I^{131} uptake

---

[2] This diet consisted of 67.5% wheat, 15% casein, 7.5% skim milk powder, 6.75% hydrogenated vegetable oil, 1% fish oil, 0.75% NaCl, 1.5% $CaCO_3$, KI added (analysis 1.0 μg. iodide per gram of diet). In addition the rats were fed lettuce once weekly.

[3] In a series of experiments employing large numbers of surgically thyroidectomized rats, done in collaboration with Dr. Alvin Taurog, the entire developmental path of the thyroid was removed after the injection of I^{131}. This continuous tissue mass which in-

determined directly in a scintillation counter. The difference between radioactivity per minute and that in an equal mass of leg musculature gave the counts per minute taken up by any thyroid remnant. The uptake was then expressed as the percentage of the total quantity of radioactivity administered. All rats in which tissue from the neck region incorporated more than 0.33% of that of the comparable region of the normal rat were considered incompletely thyroidectomized and rejected. It should be pointed out that this criterion for completeness of thyroidectomy is not adequate for the highest dosage of thyroxine, due to inhibition of the iodide concentrating activity of remaining thyroid fragments.

At autopsy the pituitary, ovaries, uterus, adrenals and thymus were removed from each rat and weighed. The pituitaries were fixed in sublimate-formol (9:1) and after nitrocellulose embedding were sectioned at four micra in a horizontal plane. Representative sections from three levels of the gland were stained by the periodic acid-Schiff-iron hematoxylin-orange G technique. The ovaries were fixed in Bouin's fluid, embedded in paraffin, sectioned serially at eight micra and stained with hematoxylin and eosin. The adrenals were fixed in 10% formol, embedded in gelatin, sectioned at ten micra with the freezing microtome and stained with Sudan black.

RESULTS

Growth

The body weight increases and final body lengths of rats in the different groups are shown in Figure 1. The growth of the untreated thyroidectomized rats had almost ceased 16 days after the operation. The lowest dose of thyroxine (0.25 μg.) supported nearly normal growth throughout the experimental period. At the 0.5 μg. level growth was definitely within normal limits. This maintenance of growth by such low levels of thyroxine is remarkable, particularly since the dosages were held constant and not adjusted to the increasing body weight. It was concluded that 0.5 μg. of thyroxine daily was necessary to maintain normal growth for 96 days from the time of thyroidectomy at 28 days of age.

Calorigenesis

The results on growth were in marked contrast to those on heat production as shown in Figure 2. Metabolic rate was considerably below normal at all dosages of thyroxine except 5 μg. Terminally there was little difference between the metabolic rate of the thyroidectomized controls and the rats receiving 0.25 and 0.5 μg. of thyroxine. It was concluded that doses of

---

cluded the base of the tongue, hyoid bone, pharynx, larynx, trachea, oesophagus, pretrachial muscles, thymus, heart, root of lungs, and all adherent tissues was divided into counting vials and the I^{131} concentration in each region determined in a scintillation counter. I^{131} concentrating tissue in rats which were incompletely thyroidectomized was only found in the thyroid region of the neck and was invariably associated with the presence of I^{131} thyroxine in the plasma as revealed by chromatography. Based on these results, the region taken for determination of completeness of thyroidectomy in the present experiment allowed a wide margin of safety.

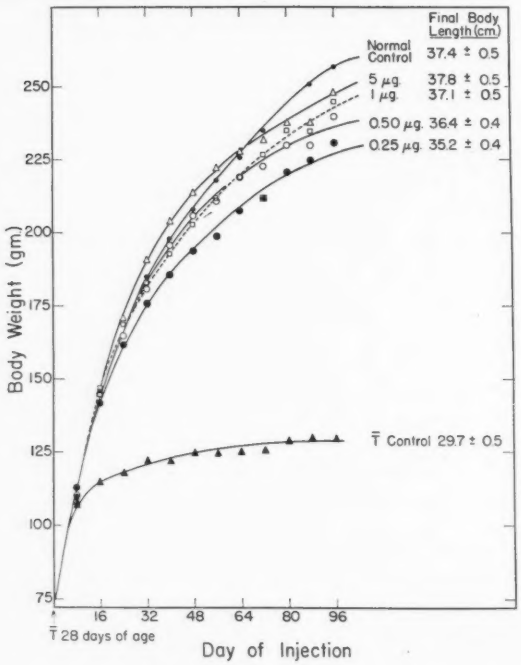


FIG. 1. Degree of maintenance of body growth of thyroidectomized rats by graded doses of L-thyroxine. The number of rats at each point on these curves was the same as the final numbers shown in Table 1. The reduction in numbers from the original groups of ten was due to the exclusion of rats with iodide concentrating tissue. The combined initial body length of all rats shown in this figure was 25.0 ± 0.2 cm.

thyroxine greater than 1 and less than 5 μg. were required to maintain metabolic rate at normal levels for 96 days.

Erythropoiesis

The degree of maintenance of red cell volume in the various groups is shown in Table 1. It was surprising that the 0.25 and 0.5 μg. doses of thy-

TABLE 1. DEGREE OF MAINTENANCE OF HEMATOLOGICAL VALUES OF THYROIDECTOMIZED RATS BY GRADED DOSES OF L-THYROXINE

Group	Dose, μg./day/ 96 days	No. of rats	Final body weight, gm.	Hemoglobin, gm./100 ml.	Hematocrit %	Total blood volume, ml.	Red cell volume, ml/100 gm. B.W.
T̄ Injected	0.25	7	231 ± 11*	12.5 ± 0.2	39.3 ± 0.7	10.80 ± 0.35	1.84 ± 0.05
	0.5	4	240 ± 10	13.0 ± 0.3	40.0 ± 1.3	10.86 ± 0.51	1.80 ± 0.06
	1	5	245 ± 7	12.9 ± 0.4	41.8 ± 0.8	12.60 ± 0.79	2.15 ± 0.10
	5	8	248 ± 11	13.4 ± 0.3	42.8 ± 1.0	12.62 ± 0.43	2.19 ± 0.16
T̄ Control	—	7	131 ± 4	11.8 ± 0.3	36.2 ± 0.9	6.62 ± 0.28	1.83 ± 0.08
N Control	—	6	258 ± 7	13.4 ± 0.5	45.0 ± 1.3	11.84 ± 0.54	2.06 ± 0.09

* Figures preceded by ± in the tables are the standard errors of the means.

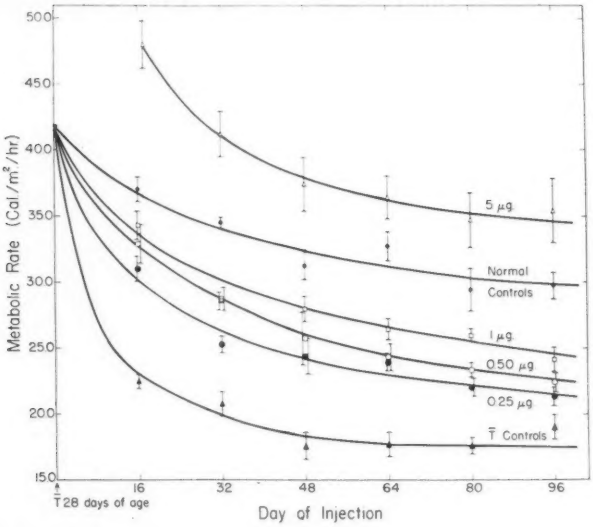


FIG. 2. Degree of maintenance of calorigenesis of thyroidectomized rats by graded doses of L-thyroxine. The number of rats at each point on these curves was the same as the final numbers shown in Table 1. The vertical bars extending from the points on the curves represent the standard errors of the means.

roxine had no ameliorative action on the characteristic decrease in red cell volume which occurs after thyroidectomy, since these levels did maintain calorigenesis to some extent. It was concluded that 1 μg. of thyroxine was required for maintenance of normal red cell volume for 96 days.

Endocrine organs

The pituitaries of rats receiving 0.25 μg. of thyroxine showed a limited maintenance of acidophils and no prevention of the hyperplasia and hypertrophy of the basophils. There was only a slight improvement in the anterior lobe after the administration of 0.5 μg. of thyroxine. The pituitaries of rats receiving 1 μg. contained normal numbers of well granulated acidophils. The basophils were still increased in number; however, they were less swollen, showed little or no vacuolation, stained more intensely, and contained fewer "T" granules. At 5 μg. the anterior lobe was normal in appearance. The deficient pituitary weight, which characterizes the rat thyroidectomized when immature, was improved by all doses of thyroxine (Table 2). It was concluded that 1 μg. of thyroxine daily was necessary for maintenance of acidophils for 96 days. Partial maintenance of basophils was definite at 1 μg. but doubtful below this level.

The ovaries of the untreated thyroidectomized rats contained a few old corpora lutea of different ages, indicating that occasional ovulation had

TABLE 2. DEGREE OF MAINTENANCE OF ENDOCRINE ORGAN WEIGHTS OF THYROIDECTOMIZED RATS BY GRADED DOSES OF L-THYROXINE

Group	Dose, μg./day/ 96 days	No. of rats	Final body weight, gm.	Pituitary, mg.	Ovaries, mg.	Uterus, mg.	Adrenals, mg.	Thymus, mg.	20 hr. I^{131} uptake* ventral neck region
$\bar{T}$ Injected	0.25	7	231 ± 11	11.2 ± 0.6	49 ± 4	386 ± 56	41 ± 3	165 ± 20	0.03
	0.5	4	240 ± 10	13.8 ± 1.3	53 ± 4	428 ± 40	46 ± 2	169 ± 9	0.02
	1	5	245 ± 7	12.7 ± 0.7	68 ± 9	447 ± 23	61 ± 2	145 ± 20	0.02
	5	8	248 ± 11	11.5 ± 0.8	66 ± 3	479 ± 51	82 ± 3	220 ± 15	0.01
$\bar{T}$ Control	—	7	131 ± 4	9.8 ± 0.4	29 ± 3	232 ± 34	23 ± 1	98 ± 12	0.04
N Control	—	6	258 ± 7	12.3 ± 1.0	76 ± 8	492 ± 90	72 ± 6	220 ± 21	18.08

* Expressed as per cent of injected dose.

occurred. Only a few small to medium follicles were present and these were usually undergoing atresia. The interstitial tissue was of variable degrees of deficiency but not as completely atrophic as in the hypophysectomized rat. The 0.25 μg. level of thyroxine maintained all components of the ovary in nearly normal condition although the weight of the organ was still subnormal (Table 2). Most ovaries showed evidence of recent ovulation or very recent corpora lutea. At the 0.5 μg. dosage the ovary appeared histologically to have attained normality rather recently although the weight was still deficient; all ovaries contained a set of newly formed corpora lutea which were 24 to 48 hours old. In addition to these, only old degenerate corpora lutea were present together with a few follicles of small to small-medium size. The interstitial tissue was normal and showed vigorous mitotic activity. The ovaries of rats receiving 1 and 5 μg. of thyroxine were normal in weight and histological structure. They contained several crops of corpora lutea of different ages and follicles varying in size in different ovaries. The interstitial tissue was normal and variable in amount. It was concluded that 1 μg. of thyroxine was required for normal ovarian function under these conditions. This conclusion, based on ovarian weight and histology, was further supported by uterine weights (Table 2).

The adrenals of the untreated thyroidectomized rats showed typical atrophy. The cortex was narrow with small cells containing uneven lipid droplets, coarser than normal. The glomerulosa was wider than normal and contained the coarsest lipid. The fasciculata was greatly reduced in width and the normal columnar arrangement of cells was ill-defined. The reticularis appeared to be less markedly affected than the fasciculata; however, it was narrower than normal and formed an abrupt junction with the medulla. The adrenals of the rats receiving 0.25 μg. of thyroxine were partially maintained, judged by histology. The cells, although larger, were not normal in size. The glomerulosa was still wider than normal. The fasciculata and reticularis were wider than in the thyroidectomized control and in some cases showed a normal lipid pattern. The increased width of the reticularis was particularly noted. The weight of the glands was maintained at almost twice that of the thyroidectomized control (Table 2). The adrenals of the rats receiving 0.5 μg. were not notably different in weight or histology from those on the lowest dosage. The glomerulosa was of normal width in some

glands and the reticularis was no longer out of proportion. The adrenals of the rats receiving 1 μg. of thyroxine showed a further degree of maintenance. Some glands were within normal limits histologically but the fasciculata and reticularis were usually narrower than normal and the weight of the gland was still subnormal. The 5 μg. level produced some adrenal cortical hypertrophy which was particularly noticeable in a widening of the reticularis. It was concluded that doses of thyroxine greater than 1 and less than 5 μg. were necessary for maintenance of normal adrenal weight and histology for 96 days.

The characteristic involution of lymphoid tissue which occurs in the immature rat after thyroidectomy was well shown by the reduced weight of the thymus of the untreated thyroidectomized controls (Table 2). Thymic weight, although increased by all levels of thyroxine, was maintained at normal only by the highest dose.

DISCUSSION

The results show that growth was the most sensitive of the various endpoints for L-thyroxine. This finding is consistent with the concept of Griesbach and Purves, based on pituitary cytology, that there is a striking biological differential in respect to the action of thyroxine. It also supports the use by this laboratory of growth as the most sensitive indicator of incomplete thyroidectomy. It has only been in recent years with the development of scintillation tubes and improved chromatographic techniques that this criterion has been exceeded in sensitivity (unpublished observations in collaboration with Dr. Alvin Taurog). In agreement with the conclusion of Scow and Simpson (1), the present results show that determination of metabolic rate gives no assurance as to the completeness of thyroidectomy since this index is very insensitive to small fragments of thyroid or to small amounts of exogenous thyroxine.

It was surprising that normal growth occurred at levels of thyroxine which did not maintain normal numbers or normal granulation of pituitary acidophils. Since the pituitaries were not assayed for growth hormone no statement can be made as to their content. Solomon and Greep (10) have recently reported that they were unable to show a quantitative relation between the degree of acidophil granulation and assayable growth hormone.

The assumption has been made that thyroxine promotes growth in the thyroidectomized animal both through reinstatement of growth hormone secretion by the anterior pituitary and by intensification of the action of growth hormone. Two hormonal influences which normally oppose growth are the secretions of the adrenal cortex and of the ovary. Both of these organs were structurally subnormal in animals receiving the levels of thyroxine which maintained growth.

Evidence has been presented previously to show that, in addition to

thyroxine, adrenal cortical secretions and growth hormone are important in determining levels of heat production (11, 12). In the present experiment the degree of maintenance of calorigenesis paralleled the structural condition of the adrenal cortex.

The parallel between oxygen consumption and red cell volume was not as evident. The red cell volume of the thyroidectomized controls did not decline to hypophysectomy levels (1.40 ml./100 gm. B.W.) although oxygen consumption had fallen to the level characteristic of hypophysectomy two weeks after thyroidectomy. The lower levels of thyroxine (0.25 and 0.5 μg.) which influenced heat production had no effect on the anemia of thyroidectomy. The higher levels which had a more marked effect on calorigenesis did restore the red cell volume to normal levels or above.

Experiments are now in progress to determine the minimal dosage of thyroxine and triiodothyronine which will produce a measurable effect on growth. The results thus far indicate that 0.05 μg. of L-thyroxine still has a marked effect on growth and that doses of 0.2 μg. and below of L-triiodothyronine are no more potent than the same levels of L-thyroxine. It has been found that high levels of injected potassium iodide will reinstate growth in the completely thyroidectomized rat (unpublished observations). It will be interesting to compare this response with that obtained from minimal doses of thyroxine and triiodothyronine.

REFERENCES

1. Scow, R. O. and M. E. Simpson: *Anat. Rec.* **91**: 209. 1945.
2. Purves, H. D. and W. E. Griesbach: *Brit. J. Exper. Path.* **27**: 170. 1946.
3. Griesbach, W. E. and H. D. Purves: *Brit. J. Exper. Path.* **26**: 13. 1945.
4. Crafts, R. C. and H. A. Meineke: *Am. J. Clin. Nutrition* **5**: 453. 1957.
5. Jacobson, L. O. and E. Goldwasser: Homeostatic Mechanisms. Brookhaven Symposia in Biology, No. 10, 1958, p. 110.
6. Evans, E. S., L. L. Rosenberg and M. E. Simpson: *Anat. Rec.* **133**: 273. 1959.
7. Kleiber, M.: *Univ. Calif. Publ. Physiol.* **8**: 207. 1940.
8. Berlin, N. I., R. L. Huff, D. C. Van Dyke and T. G. Hennessy: *Proc. Soc. Exper. Biol. and Med.* **71**: 176. 1949.
9. Turner, A.: *Bull. U. S. Army Med. Dept.* **5**: 605. 1946.
10. Solomon, J. and R. O. Greep: *Endocrinology* **65**: 158. 1959.
11. Evans, E. S., A. N. Contopoulos and M. E. Simpson: *Endocrinology* **60**: 403. 1957.
12. Evans, E. S., M. E. Simpson and H. M. Evans: *Endocrinology* **63**: 836. 1958.

BLOOD AND URINE CALCIUM CHANGES IN TOTALLY PARATHYROIDECTOMIZED *RANA PIPIENS*[1]

JOHN R. CORTELYOU, AILEEN HIBNER-OWERKO AND JOSEPH MULROY

Department of Biological Sciences, De Paul University, Chicago 14, Illinois

ABSTRACT

The effects of parathyroidectomy in *Rana pipiens* are being investigated in our laboratories. The results of the work being reported demonstrate that the calcium concentration in whole blood and plasma gradually decrease 50 to 60% below the control values within 1 to 2 weeks. However, a hypocalcemia is not permanent as is evidenced by the fact that at the end of 29 weeks the whole blood calcium of the operated animals returned to 8.3 mg.% which approximates the lowest calcium values obtained from unoperated animals. Calcium concentrations in urine taken from the parathyroidectomized animals on different days are 13 to 78% above the control values. These results indicate that in parathyroidectomized frogs there is a loss of calcium through the urinary system which is concomitant with the decrease in plasma calcium. No death from tetany occurred in the operated animals even when their blood or plasma calcium was at its lowest levels. Histological examination of parathyroid glands taken from winter frogs revealed that these glands undergo a degeneration wherein the tissue is highly reticular and the cells appear to be degenerating.

LITTLE is known concerning the role of the parathyroid glands in amphibians. In 1888, Maurer (1) termed these small bodies "epithelkorperchen." He found them to originate from the third and fourth branchial arches during the larval stage of the frog and during metamorphosis in *Triton taeniatus*.

Baldwin (2) and Scholz (3) described the anatomy and histology of these glands in several species of Amblystoma. Descriptions have been given for the parathyroid glands of anurans by Shapiro, (4), Romeis (5) and Waggener (6).

Bilaterally parathyroidectomized *Salamandra atra* and *S. maculosa* were observed by Scholz (3) to remain alive for one year with no evidence of tetany. Total parathyroidectomy of *Rana temporaria* (5) and *Hyla aurea* (7) did not result in tetanic responses or death. However, Waggener (8) reported mild to severe tetanus followed by death in approximately 66 hours in parathyroprivic *Rana catesbiana*. Associated with tetany he reported a decrease in blood calcium from 11.5 to 7.5 mg.%.

It was thought appropriate to resume studies on amphibian parathyroid

Received October 2, 1959.

[1] Supported by the National Science Foundation; Grant No. G5670.

gland functions with a view to a more extensive investigation of their role as an endocrine gland in lower vertebrate animals. Such information will permit a comparison with the parathyroid gland function in birds and mammals and may shed some light on the analysis of parathyroid action.

MATERIALS AND METHODS

Large male *Rana pipiens* used in these studies were obtained from Northern Wisconsin and were maintained on a water table with a constant flow of aerated water at a temperature of 18–22° C. Operated animals were confined in individual perforated containers.

Parathyroid glands were removed from ether anesthetized animals on moist cheese cloth. With an iridectomy scissors a small "V" shaped skin incision was made at the junction of the coracoid and sternum, thereby forming a movable skin flap. With jewelers forceps the subhyoid muscle and fascia were separated from the deltoid muscle. Connective tissue at the episternal angle was cleared away to expose the external jugular vein, the hypoglossal nerve and the omohyoid muscle. The omohyoid was gently pulled in an antero-lateral direction so as to expose the large highly vascularized kiemenrest and the parathyroid glands. The kiemenrest serves as a reference point to locate the parathyroids. The latter were removed by gently pulling them free from their connective tissue attachment. All operations were made with a dissecting microscope at 10 diameters magnification.

Tissue to be examined histologically was prepared by employing the usual procedures for embedding and sectioning. Sections were cut at 4 to 8 micra and Heidenhains or Delafields hematoxylin with eosin were used as stains. Microscopic observations and photomicrographs were made at 100 and 860 diameters.

Blood samples were obtained by means of heart puncture with heparinized syringes and were transferred to either 3 ml. test tubes or 0.2 ml. hematotubes, dependent on whether whole blood or plasma was to be analyzed. When plasma was to be used the hematocrit tubes were placed in a micro hematocrit centrifgue for 6 minutes. The portion of the tube containing the clear plasma was cut and the end capped with critocaps.

Calcium determinations of whole blood were made according to the method of Roe-Kahn (9) and readings were made with a Rouy-Leitz photometer. Plasma and urine calcium determinations were made by flame analysis at a wave length of 422.8 mμ. with a Beckman DU spectrophotometer and photomultiplier attachment. Hydrogen served as a fuel source instead of acetylene. It is our experience that more consistent results are obtained with hydrogen.

Calcium standards for the analysis of blood were prepared with either Lab-trol or Patho-trol (Dade Reagents). Standards and blood samples were prepared in dilutions of 1:50 using 50 μl. of each sample brought to 2.5 ml. of triply glass distilled water. Standards for the analysis of urine calcium were also prepared with these reagents and the urine samples were not diluted.

It was not necessary to use a catheter to obtain the urine samples. The frogs were gently removed from the water at which time there was a small amount of urine eliminated. Each frog was held vertically with the dorsal surface of the cloacal opening facing a wide mouthed funnel set in a 15 ml. test tube and the lateral abdominal walls were gently compressed. A stream of clear urine jetted forth into the funnel and was collected in the tube. The funnel was held such that there was no dilution by body water from the animal.

Sciatic nerve-gastrocnemius preparations were isolated from control and parathyroidectomized frogs. Recordings were made of responses to stimuli introduced at graded frequencies by means of an Electrodyne stimulator.

RESULTS

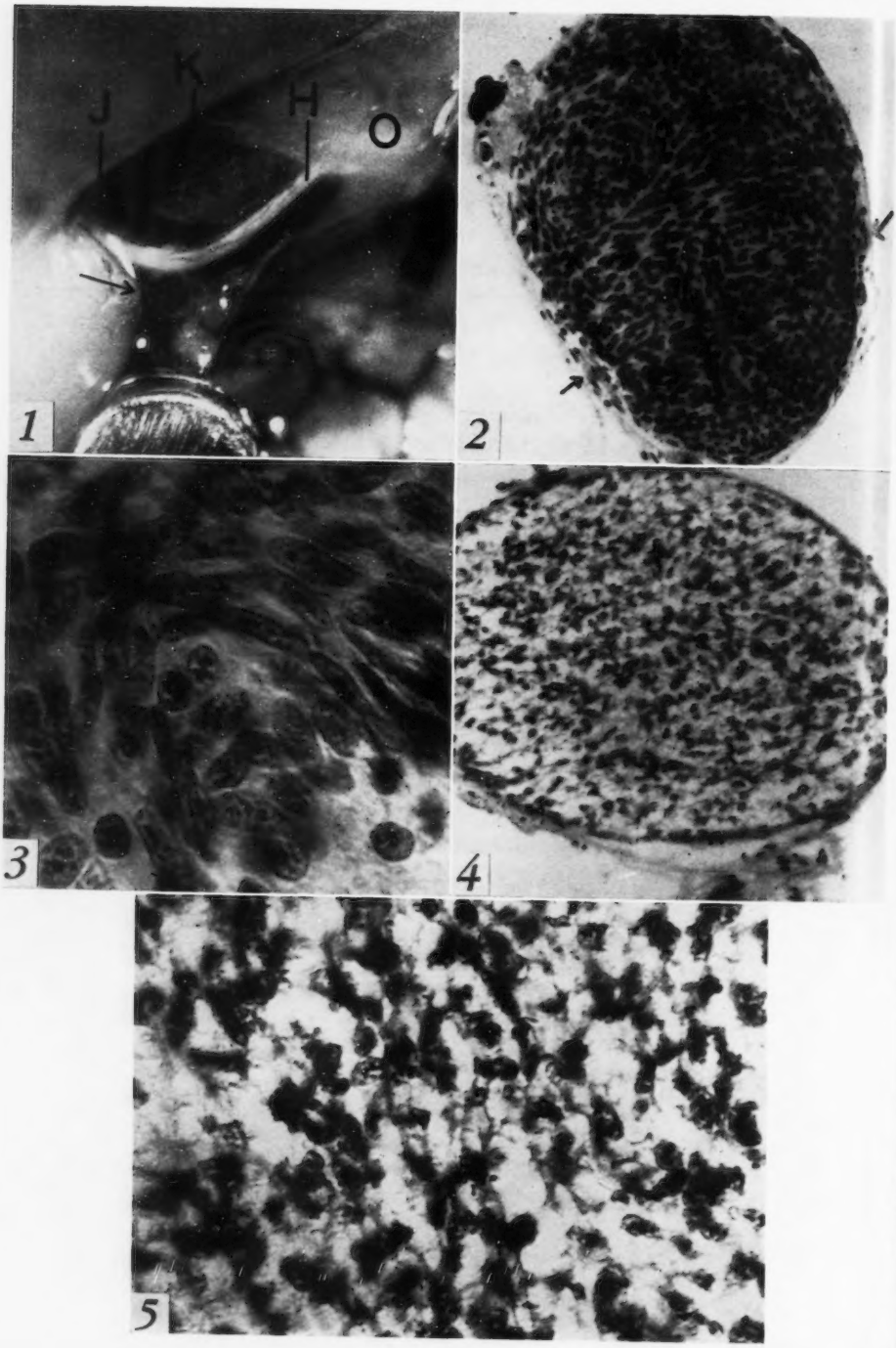
The two pairs of parathyroid glands in *R. pipiens* lie lateral to the external jugular vein and dorsal or dorso-lateral to the hypoglossal nerve (Fig. 1). Medial to or parallel with the parathyroids is a larger and more highly vascularized structure, the kiemenrest (Fig. 1). The more anterior location of the thyroid glands in these animals avoids the necessity of thyroparathyroidectomy.

Cells of these glands are epithelial, arranged in whorls and are confined within a thin connective tissue capsule below which is a dense capillary network (Fig. 2). Those cells arranged in whorls are elongated and are usually located more centrally (Fig. 3). Glands taken from frogs between October and December appeared to lose their structural integrity as evidenced by a distinct reticular appearance (Fig. 4). Though there is not a complete absence of cellular elements, remaining cells are extremely atypical and appear to be degenerating (Fig. 5). Cyclic changes in the cellular organization of amphibian parathyroids have also been observed by Romeis (5) and Waggener (6).

No death from tetanic convulsions occurred in parathyroidectomized frogs nor in three hundred such animals was there any evidence of a tetanic spasm. Though no gross symptoms of hyperexcitability were evident it seemed possible that more subtle expressions of such a condition might be found in neuromuscular preparations. Results of experiments carried out on sciatic-gastrocnemius preparations from 54 animals are summarized in Table 1. A comparison of the percent of preparations demonstrating treppe, incomplete and complete tetanus shows no significant difference between the normal and 2 to 12 week post-operative animals. The evidence indicates that 50% of the preparations taken within 5 days showed an increased

TABLE 1. FREQUENCY OF STIMULATION REQUIRED TO CAUSE TREPPE, INCOMPLETE TETANUS AND TETANUS IN A SCIATIC NERVE-GASTROCNEMIUS MUSCLE PREPARATION TAKEN FROM SHAM CONTROL ANIMALS AND TOTALLY PARATHYROIDECTOMIZED *Rana pipiens*

Condition	Treppe	Incomplete tetanus	Tetanus	Relaxation delay
Sham Controls	10–20/sec. 79%	20/sec. 71%	22+/sec. 37%	None
	5–10/sec. 21%	10/sec. 29%	21–22/sec. 63%	None
2–5 day operated animals	10–20/sec. 50%	20/sec. 50%	20–30/sec. 50%	Positive
	5–10/sec. 50%	10/sec. 50%	10+/sec. 50%	Positive
2–12 week operated animals	10–20/sec. 92%	20/sec. 69%	22–30/sec. 31%	Positive
	5–10/sec. 8%	10/sec. 31%	20–22/sec. 69%	Positive


J
K
H
O
1
2
3
4
5

excitability. Each of the motor responses was obtained at lower stimulating frequencies in more cases than in a normal group of animals. Furthermore, fusion frequency was below the normal range in the majority of animals studied within this period after the operation. By 2 weeks these responses returned to normal in the majority of cases. However, from these results it would be difficult to state that there is a characteristic response of hyperexcitability resultant from parathyroidectomy of *R. pipiens* because there is also a high percentage of the preparations from parathyroid deficient animals having excitability responses within the normal range. The only consistent suggestion of hyperexcitability is the occurrence of relaxation delay as is shown in Figures 7 and 8. Myographs in Figure 6 are typical responses obtained from preparations of a sham operated control frog. The most extreme variations from the normal response are shown in Figures 7 and 8.

Blood calcium values for control and experimental animals are given in Figure 9. To facilitate the expression of calcium changes, mean values are included. Unoperated frogs had calcium values ranging from 9.4 to 12.5 mg.% and are referred to as normal in this study. The mean calcium value for whole blood of *R. pipiens* is 10.3 mg.%.

Twenty four hours after parathyroidectomy the mean blood calcium was 7.2 mg.% or 30% lower than the controls. At 48, 72 and 96 hours the average calcium levels were 6.9, 5.6 and 3.3 mg.% respectively, representing decreases of 33.2, 45.7 and 68%. Seven day post-operative animals were 63.3% below their controls. Calcium levels in 5 and 6 day operated frogs were 5.7 and 5.5 mg.%. Although this is not as low as either the 4 or 7 day groups it is assumed that they fall within a range of variation that would be found in parathyroidectomized frogs between days 4 and 7.

After the 14th day, calcium determinations were made at weekly intervals. The values between 3 and 6 weeks closely approximate each other. However, calcium values for the 3 week operated frogs more closely approximate those for most of the animals of the 2nd week, the difference

PLATE I. EXPLANATION OF FIGURES

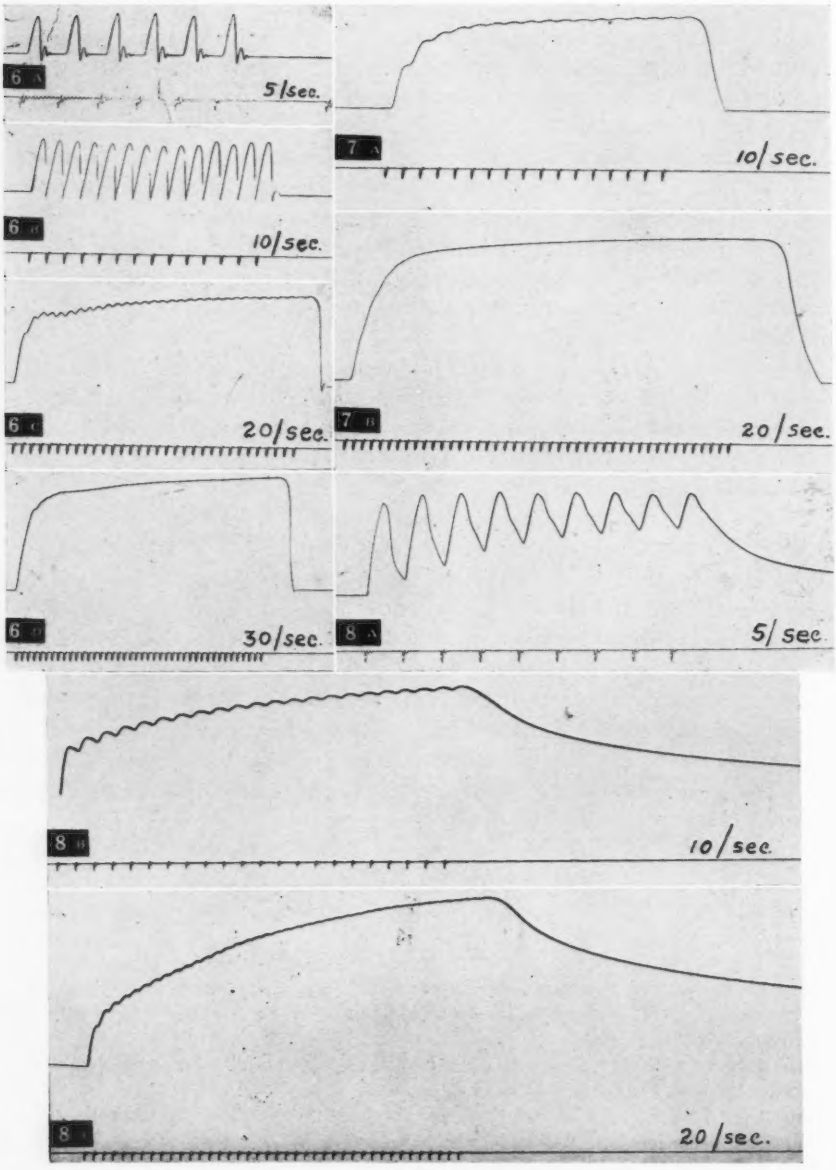
FIG. 1. Parathyroid glands in *Rana pipiens* showing the relation to kiemenrest (K), hypoglossal nerve (H), external jugular vein (J), omohyoid muscle (O). Arrows point to parathyroid glands. ×120.

FIG. 2. Section of normal parathyroid gland. Arrow points to sub-capsular capillary networks. Whorled cells centrally located. ×200.

FIG. 3. High power view of parathyroid cells, particularly the elongated cells which are more centrally located. ×860.

FIG. 4. Section of parathyroid gland removed from a winter frog which shows a recticular type of degeneration. × 200.

FIG. 5. High power view of reticular type of degeneration Note the lack of typical cells and the reticular appearance of the tissue. ×860.


6 A
5/sec.
6 B
10/sec.
6 C
20/sec.
6 D
30/sec.
7 A
10/sec.
7 B
20/sec.
8 A
5/sec
8 B
10/sec.
8
20/sec.

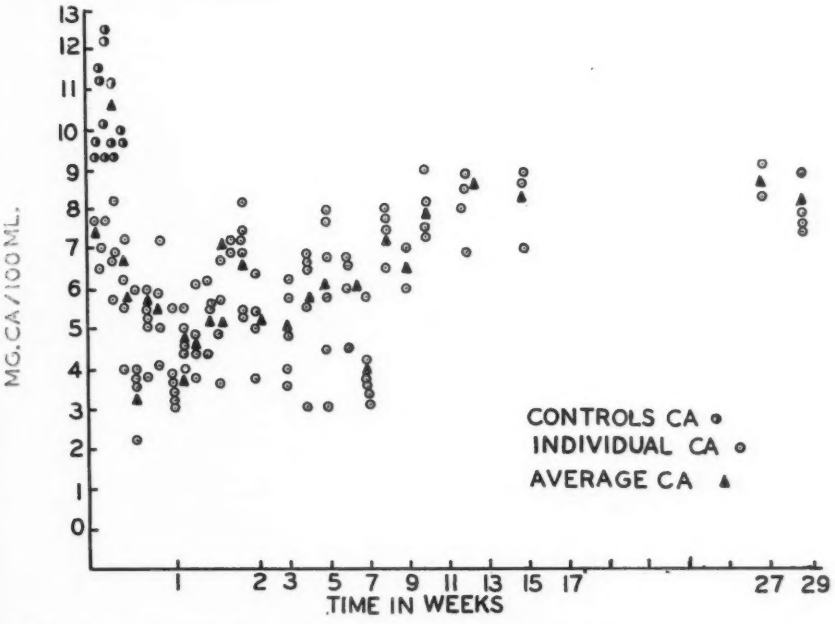


FIG. 9. Distribution of blood calcium values obtained from whole blood samples of normal and parathyroidectomized *Rana pipiens*. The decrease in blood calcium during the first post-operative week is best noted from average values (▲). A more gradual rise to the control level follows but remains sub-normal through 29 weeks.

PLATE II. EXPLANATION OF FIGURES

Myographs of sciatic-gastrocnemius preparations of normal and bilaterally parathyroidectomized *Rana pipiens*. Stimulation was accomplished with use of a thyratron pulse generator (Electrodyne, Model 461) at voltages of 5 to 10. Stimulating frequencies are designated.

FIG. 6. Records from a sham-operated control animal (#209). Immediate relaxation after stimulation is typical. Incomplete tetanus is reached at stimulating frequency of 20/sec. (C). Based on the duration between effective stimuli in this myograph (C), it is calculated that the fusion frequency would be 21 to 22 stimuli/sec.

FIG. 7. Myograph of parathyroidectomized frog (#76) at 48 hours postablation. Incomplete tetanus is observed at a stimulating frequency of 10/sec. (A). The fusion frequency is calculated to be 13 to 15 stimuli/sec. as compared with complete tetanus in controls at 21 to 22 stimuli/sec.

FIG. 8. Myograph of parathyroidectomized frog (#130) at 3 weeks postablation. Greater excitability than controls is shown where incomplete tetanus results from a stimulating frequency of 10/sec. (B); this is also evidenced by treppe occurring at 5 stimuli/sec. (A). A response of incomplete tetanus at 20 stimuli/sec. (C) is the same as occurs in controls Hyperexcitability is further demonstrated by a pronounced delay in relaxation time after stimulation (A, B, and C).

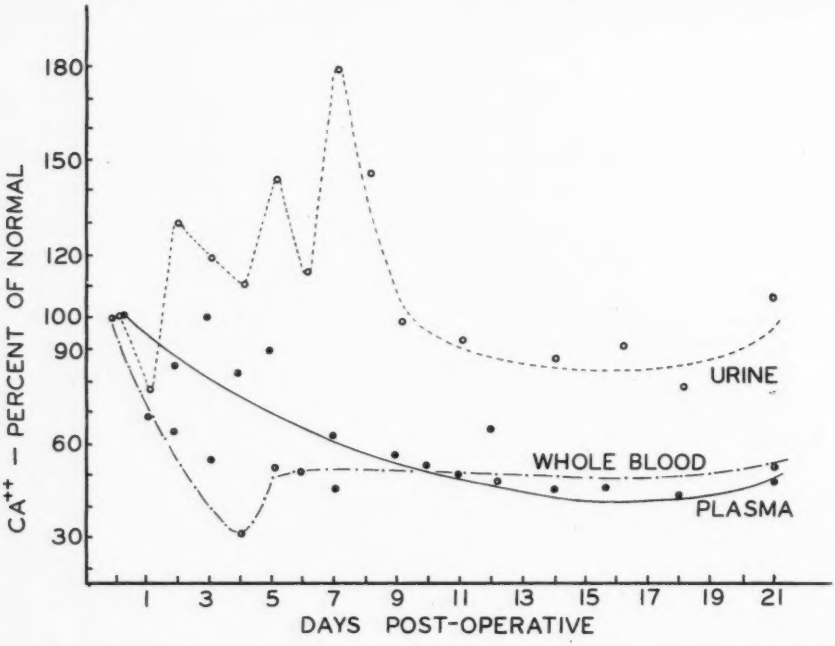


FIG. 10. Comparative analysis of the changes in calcium concentration in whole blood (Roe-Kahn method), plasma and urine (flame analysis) of totally parathyroidectomized frogs.

only being a 0.2 mg.% increase. For 4, 5 and 6 week animals there is an increase in the mean of only 0.3 mg.%. The mean calcium concentration in these animals is 5.8 mg.% which represents a 13.7% increase over 2 week post-operative animals.

Between the 8th and 29th week mean blood calcium values steadily increase. Blood samples taken from the 10th to the 29th week are similar in calcium values, showing a maximum variation of 0.9 mg.%. The mean for all of these samples is 8.3 mg.% which is 20.3% higher than that found in 8 and 9 week animals.

Plasma calcium determinations were made on another series of parathyroidectomized frogs and the experiments were terminated at the end of 3 weeks. The data are shown in Figure 10 in comparison with whole blood and urine calcium. The mean value for plasma calcium in unoperated *R. pipiens* is 6.6 mg.%. Forty eight hours after parathyroidectomy it falls to 5.7 mg.%; 13.7% below controls. At the end of 7 days it is 4.0 mg.%; 38% below the controls. These results show a gradual decrease of plasma calcium in parathyroidectomized frogs and the lowest level of plasma calcium is reached within 2 weeks after gland removal.

The mean value of normal urine calcium is 0.186 mg.% for *R. pipien*

Twenty four hours post-parathyroidectomy there is a sharp drop to 0.145 mg.% (22% below normal) followed by a marked rise in the next 24 hours to 0.240 mg.% (28% above normal). Between days 2 and 8 urine calcium values remain from 11 to 78% above normal. From days 9 to 18 the urine calcium values were 3 to 16% below those of the controls. However, by day 21 the urine again shows a 10% higher concentration of calcium than is found in the controls.

DISCUSSION

Waggener (8) has shown that parathyroprivic *R. catesbiana* have lowered blood calcium to 7.7 mg.% which results in tetany and death. Results reported in this work with *R. pipiens* show a somewhat different picture. Parathyroidectomy does not result in any gross manifestations of tetany nor in any significant changes in the direction of hyperexcitability. Whole blood calcium falls to its lowest value between 4 and 7 days. The depression occurs more slowly than that reported for parathyroidectomized rats wherein the lowest calcium values are observed within the first 24 to 48 hours (10, 11). Monahan and Freeman (12) reported serum calcium decreases in nephrectomized-parathyroidectomized dogs from 11.3 to 5.9 mg.% in 72 hours.

The lowered blood calcium in frogs is not permanent as evidenced by the fact that at the end of 29 weeks after gland removal the blood calcium was 8.3 mg.%, which approaches the lower limit of the normal range.

Tweedy and Chandler (10) observed that in parathyroidectomized rats there is a return of serum calcium in the direction of normal, but this occurs within several days to a week and only reaches the lowest values within the normal range. Thus, there is a difference between frogs and rats in the rate at which there is a reduction and subsequent elevation of calcium. In frogs this series of variations is considerably longer. The gradual rise in blood calcium is not considered to be an expression of dietary intake. Parathyroidectomized animals fed 5 meal worms a week for 7 weeks still had a low blood calcium (3.1 to 5.8 mg.%). Investigations are currently in progress to study the influences of exogenous calcium and phosphorus supplies as well as parathormone support in parathyroidectomized frogs.

Plasma calcium in *R. pipiens* is considerably lower than whole blood calcium, the mean being 6.6 mg.% with a range of 5.2 to 8.6 mg.%. As with whole blood, plasma calcium decreases in parathyroidectomized animals, and its low values are quite similar to those of whole blood. It would appear that the decline of plasma calcium is more gradual and prolonged, with the lowest values being reached at approximately 2 weeks. Studies with whole blood show the minimum value at approximately 1 week.

Hypocalciuria has been consistently reported to be a result of parathyroid deficiency in mammals (13). The data for parathyroidectomized frogs show an initial drop in urine calcium followed by an increase above normal

by the 2nd post-operative day which persists for approximately a week.The relationship between the lowered plasma calcium concentration and increased urine calcium concentration suggests that the lack of parathormone impairs the ability of the renal organ to reabsorb calcium. Whatever the mode of action of parathormone, it is significant to note that hypercalciuria does occur in parathyroidectomized frogs.

Variations in the hypercalciuric values during 7 days suggest an attempt by the animals to attain a calcium equilibrium, unsuccessful as it is, during this time. It would also appear that after blood calcium has reached its lower limits the frogs adjust to establish a blood calcium approaching normal by a mechanism other than the parathyroid glands which elevates renal threshold. Buchanan, Kraintz and Talmage (14) have recently reported an initial decrease of calcium excretion in parathyroidectomized mice, followed by a three-fold increase above normal calcium excretion. Within 14 hours the urinary excretion of calcium returned to within the normal range. These workers indicate that spectacular results in regard to parathyroid effects on renal calcium cannot be expected since its excretion rate is very low and due to excretion variability, changes are difficult to establish. It would seem that the slowness and the persistence of the changes in blood and urine calcium in parathyroidectomized *R. pipiens* may permit this animal to be a useful tool in the study of the site and mode of action of the parathormone.

REFERENCES

1. MAURER, F.: *Morph. J. B.* **13:** 296. 1888.
2. BALDWIN, F. M.: *J. Morph.* **30:** 605. 1918.
3. SCHOLZ, J.: *Zeit. f. Mikr. Anat. Forsh.* **34:** 159. 1933.
4. SHAPIRO, B. G.: *J. Anat.* **68:** 39. 1933.
5. ROMEIS, B.: *Zeit. f. d. Gesamte Anat.* **80:** 547. 1926.
6. WAGGENER, R. A.: *J. Morph.* **48:** 1. 1929.
7. KUFFLER, S. W.: *J. Physiol.* **103:** 403. 1945.
8. WAGGENER, R. A.: *J. Exp. Zool.* **57:** 13. 1930.
9. ROE, J. H. AND B. S. KAHN: *J. Biol. Chem.* **81:** 1. 1929.
10. TWEEDY, W. R. AND S. B. CHANDLER: *Am. J. Physiol.* **88:** 754. 1929.
11. TALMAGE, R. V., F. W. KRAINTZ, R. C. FROST AND L. KRAINTZ: *Endocrinology* **52:** 318. 1953.
12. MONAHAN, E. P. AND S. FREEMAN: *Am. J. Physiol.* **142:** 104. 1944.
13. GREEP, R. O.: In: The Hormones. Pincus, G. and K. Thimann, Eds. **1:** 255. Academic Press, 1948.
14. BUCHANAN, G. D., F. W. KRAINTZ AND R. V. TALMAGE: *Proc. Soc. Exper. Biol. and Med.* **101:** 306. 1959.

EFFECT OF MAGNESIUM ION ON THE RESPONSE OF THE RAT UTERUS TO NEUROHYPOPHYSIAL HORMONES AND ANALOGUES[1]

ROBERT A. MUNSICK[2]

Department of Pharmacology, College of Physicians and Surgeons, Columbia University, New York

ABSTRACT

Absolute oxytocic potencies of highly purified neurohypophysial hormones and analogues were determined on isolated rat uteri suspended in van Dyke-Hastings solution. Assays were performed in bath solutions with and without magnesium ions. In a concentration of 0.5 mM/l. magnesium ions were found to augment the response to the U.S.P. Standard, oxytocin, lysine and arginine vasopressins and all the analogues tested, but there was considerable variation between substances in the degree to which this potentiation occurred. Advantages inherent to the solution of van Dyke and Hastings are discussed, and reasons are presented for utilizing this solution without magnesium for assays of oxytocin and posterior pituitary extracts.

DALE'S discovery of the oxytocic action of posterior pituitary extract (1) was followed in 1913 by Dale and Laidlaw's description of a method of assaying such extracts on isolated, virgin guinea pig uteri (2). These and subsequent investigators have commented on modifications of the uterine response to posterior pituitary extracts by alterations of the ionic composition of the bath fluid. The divalent cations calcium and magnesium have received major consideration.

Kochmann (3) first showed that diminution of calcium or increase of magnesium concentration could cause quiescence of the isolated guinea pig uterus, and the latter observation was confirmed by Burn and Dale (4). Although subsequent investigators have endorsed various magnesium concentrations, most have agreed that 0.5 mM/l. calcium is satisfactory.

Potentiation by magnesium of the oxytocic action of posterior pituitary extract on the guinea pig uterus was first reported by van Dyke and Hastings (5). They used a solution which replicated an ultrafiltrate of guinea pig plasma (Table 1) (5, 6). Raising the calcium concentration above 0.53 mM/l. caused spontaneous contractions and increased responsiveness to posterior pituitary extract, whereas lower concentrations di-

Received October 5, 1959.

[1] Supported by research grant H1788 from the National Heart Institute, National Institutes of Health.

[2] Josiah Macy, Jr. Foundation Fellow, Dept. of Obstetrics and Gynecology, assigned to the Department of Pharmacology.

minished responsiveness. Magnesium in a concentration of 1.0 mM/l. augmented responses, but increasing its concentration to 3.0 mM/l. decreased responses. Genell (7) extended these observations to other species, using Pitocin in lieu of whole posterior pituitary extract.

Fraser investigated commercial vasopressor and oxytocic fractions of the posterior pituitary, and found that the response to the vasopressor fraction was augmented far more than the oxytocin response when magnesium ions were present in van Dyke-Hastings solution (8). Stewart reported that magnesium potentiated responses to Pitocin and to Pitressin but that the degree was variable and did not correlate well with magnesium concentration (9).

In vivo potentiation by magnesium of the uterotonic action of Pitressin and Pitocin in nonpregnant cows has been studied by Fitzpatrick (11). A

TABLE 1. ELECTROLYTE COMPOSITION OF SOME SOLUTIONS USED FOR OXYTOCIC ASSAYS. ALL CONCENTRATIONS IN mM/l. UNLESS OTHERWISE NOTED

Solution	Ca^{++}	Mg^{++}	Na^{+}	K^{+}	Cl^{-}	HCO_3^{-}	$H_2PO_4^{-}$ $HPO_4^{=}$	pCO_2, mm.Hg	pH	Glucose, mg.%
1. Dale and Laidlaw (2)	2.2	0	160	5.6	164	6.0	—	—	—	100
2. Burn and Dale (4)	2.2	0.052	160	5.6	164	6.0	—	—	—	50
3. British Pharmacopoeia (10) (1948)	2.2	0.026	160	5.6	164	6.0	—	—	—	50
4. Beauvillain (12)	0.54	0.052	160	5.6	161	6.0	—	—	—	50
5. Burn, Finney & Goodwin (15)	0.54	0	160	5.6	161	6.0	—	—	—	50
6. van Dyke and Hastings (5)	0.53	1.0	144	6.2	122	30	1.0	46	7.4	—
7. Present modification of (6)	0.50	0 / 0.50	144	6.2	122	30	1.0	40	7.4	50

three-fold increase in plasma magnesium concentration caused definite potentiation of the response to Pitressin, but not to Pitocin.

Although Beauvillain first advocated the use of rat uteri for oxytocic assays (12), it was Holton's work which convincingly demonstrated the advantages inherent to the oxytocic assay employing the rat uterus in lieu of the guinea pig uterus (13). She used the four-point assay design of Schild (14). Burn, Finney, and Goodwin present her method almost unmodified, and advocate a magnesium-free Beauvillain solution (Table 1) (15).

Until du Vigneaud and coworkers purified oxytocin (16), arginine vasopressin (17) and lysine vasopressin (18) only partially purified fractions of mammalian neurohypophysial hormones were available for study. Use of commercial posterior pituitary fractions is now known to involve two drawbacks: first, contamination of pressor with oxytocic hormone and *vice versa*, and second, use of both ox and pig posterior pituitaries for the vasopressor fraction. Arginine vasopressin from oxen and lysine vasopressin from pigs vary in pharmacological properties (19, 20). Since neither the oxytocic potency nor the effect of magnesium on the oxytocic potency of lysine vasopressin has been wittingly studied, spurious data may have arisen from use of lysine vasopressin or a mixture of lysine and arginine vasopressins. Highly purified arginine vasopressin has been shown to have a pressor/

oxytocic ratio of 20/1 (21). It was assayed in the magnesium-free bath solution recommended by Burn, Finney and Goodwin (22, 15).

The following study is a portion of an investigation of the structure-activity relationship of neurohypophysial hormones and some of their analogues, all of which were generously provided by Professor du Vigneaud in a highly purified state.

MATERIALS AND METHODS

1. *Neurohypophysial hormones and their analogues.* These highly purified polypeptides were carefully desiccated and weighed by Professor du Vigneaud's group at the Cornell University Medical College and were dissolved at this laboratory in dilute acetic acid at pH 2.9–3.0, containing 154 mM/l. sodium chloride and 0.5% chlorobutanol. The solutions were placed in ampoules, sealed, placed in a boiling water bath for ten minutes and refrigerated at 4° C until assayed. To prepare a solution for assay the contents of an ampoule were diluted with 154 mM/l. NaCl solution if the dilution was 1/20 or greater or were brought to pH 7.2–7.4 with 154 mM/l. $NaHCO_3$ solution and further diluted with 154 mM/l. NaCl solution if less than a 1/20 dilution was desired.

2. *U.S.P. Posterior Pituitary Standard.* This was the reference standard used at all times. Its potency was 88% of the International Posterior Pituitary Standard as measured by rat pressor and rabbit milk-ejection assays.

3. *Bath Solution.* The bath solution employed was a modified van Dyke-Hastings solution (Table 1). It was chosen because of its isosmoticity, its physiological pH and ionic composition, its excellent buffering capacity and the ease with which alterations in magnesium concentration could be achieved without disturbing osmoticity. The calcium concentration employed was 0.5 mM/l.

Potencies in bath environments containing magnesium concentrations of 0.0 and 0.5 mM/l. are compared. Uteri were nearly always quiescent in either solution. The reservoir and 10 ml. bath chamber solutions were gassed with oxygen containing 5% CO_2. Phenol red (0.0001%) in the solution allows the assayist to observe pH within 0.1 unit continuously. pH's of several batches of solution were determined and were always 7.4–7.5.

4. *Assay.* Assays were conducted exactly as outlined by Holton (13). The uteri were obtained from young, virgin rats, usually of the Long-Evans strain, which weighed 150–200 gm. Each unknown was assayed on at least two different uteri with and without magnesium. The range of working concentrations of USP Standard in the 10 ml. bath was 50–600 μU./ml.

5. *Statistical evaluation of results.* Data were computed by the method outlined by Holton (13). Several six-point assays were performed.[3] Estimates of potency were combined by the method of Finney (23) ignoring the magnitude of g and significant differences in estimates. Most g values were less than 0.05, and except for those assays in which the slopes of regression of unknown and standard departed significantly from parallelism, very few estimates were significantly disparate. In those assays where a significant deviation from parallelism existed the range of estimates rather than the 95% fiducial limits is tabulated, and the mean potency is represented by the weighted mean. The 5% level of probability was used to test significance of all data.

[3] The author wishes to express his thanks to Dr. George L. Saiger, Department of Biostatistics, College of Physicians and Surgeons, for his aid in computing the six-point assays.

RESULTS

All results are expressed as U.S.P. U/mg. of hormone or analogue in Table 2. It should be noted that lysine vasopressin was the sole substance tested whose slope of regression departed significantly from parallelism to the slope of regression of the U.S.P. Standard. It did so only in the presence of magnesium. Valyl oxytocin,[4] an analogue of oxytocin which is not pure, was also assayed in both solutions. Results are expressed as U.S.P. U/ml. of ampouled solution labeled "OTV-80". Table 2 also shows the highest potency of each substance from rat-pressor, intravenous dog-antidiuretic, rabbit milk-ejection, avian depressor and rat-oxytocic assays. The ratio

$$\text{U.S.P. units} \frac{\text{0.5 mM/l. Magnesium}}{\text{0.0 mM/l. Magnesium}}$$

is also shown in Table 2.

Absolute responses to the U.S.P. Standard, oxytocin, the vasopressins and all analogues tested were invariably augmented by magnesium.

A Beauvillain solution was prepared for the purpose of studying pH. Three aliquots were treated as follows: 1) gassed with oxygen containing 5% CO_2, 2) gassed with air and 3) ungassed and unstoppered in a volumetric flask. At the end of one and one-half hours the pH of the first was 6.8, of the second was 8.3 and of the third was 7.9.

DISCUSSION

No discussion of the absolute oxytocic potencies of neurohypophysial hormones or their analogues seems necessary; the values have been presented in Table 2. Potentiation of oxytocic responses to all the substances assayed in the presence of magnesium compared with responses in the same solution without magnesium confirms and amplifies the findings of previous investigators who used whole posterior pituitary extracts or commercial fractions of them. Magnesium evidently potentiates the uterotonic action of oxytocin less than it potentiates any other polypeptide tested. Potentiation of the response to arginine vasopressin causes an apparent slightly lower potency of oxytocin in a solution containing magnesium compared with its potency in one not containing this important divalent cation. This is due entirely to the increased contribution of arginine vasopressin to the oxytocic potency of U.S.P. Standard, which causes the Standard to be relatively more potent and oxytocin *by comparison* to be less potent.

To obtain a maximal value for the absolute potency of oxytocin, one should use an assay which is completely insensitive to the arginine vasopressin of the standard. Such an assay has not been described. Of the assays generally employed for oxytocin, the potencies of arginine vasopressin are as follows: rat uterus, without magnesium (9 U/mg.), rat uterus, 0.5 mM/l.

[4] Generously provided by Dr. R. Bircher of Sandoz Pharmaceuticals, Inc., Hanover N. J.

TABLE 2. OXYTOCIC POTENCIES OF NEUROHYPOPHYSIAL HORMONES AND ANALOGUES

Hormone or analogue			U/mg. & 95% fid. limits		Ratio $\frac{0.5}{0.0}$ Mg.$^{++}$	Maximal potency	
			0 Mg.$^{++}$	0.5 Mg.$^{++}$		u./mg.	Assay*
Oxytocin	CyS.Tyr.Ileu.Glu(NH_2).Asp(NH_2).CyS.Pro.Leu.Gly(NH_2)		420 (390–450)	360 (330–380)	0.86	420	R.U.
Oxypressin	.Tyr.Phe.	.Leu.	16 (15– 17)	22 (20– 23)	1.4	89	M.E.
2-Phenylalanine	.Phe.Ileu.	.Leu.	27 (25– 28)	77 (74– 85)	2.9	82	M.E.
Valyl Oxytocin[1]	.Tyr.Val.	.Leu.	1.8 (1.6–1.9)	6.4 (6.0–6.9)	3.6	7.4	M.E.[2]
Arginine Vasopressin	.Tyr.Phe.	.Arg.	9.1 (7.6– 11)	15 (14– 16)	1.6	300	R.P. & A.D.H.
Lysine Vasopressin	.Tyr.Phe.	.Lys.	5.1 (4.5–5.7)	13 (9.3– 25)[3]	2.6	200	R.P.
Histidine Vasopressin	.Tyr.Phe.	.His.	—	7.3 (6.1–8.8)	—	18	M.E.
Arginine Vasotocin	.Tyr.Ileu.	.Arg.	37 (34– 41)	71 (65– 78)	1.9	100	A.D.

[1] u./ml.
[2] Only other assay performed.
[3] Range, not fiducial limits.
* R.U.—Rat uterus, 0.0 mM/l magnesium; M.E.—Rabbit milk ejection; R.P.—Rat pressor; A.D.H.—Dog antidiuretic; A.D.—Avian depressor.

magnesium (15 U/mg.), avian depressor (42 U/mg.) and rabbit milk-ejection (51 U/mg.) (20). Hence, the greatest absolute value for the potency of oxytocin will be found in the rat oxytocic assay in the absence of magnesium, and for assays of neurohypophysial extracts this assay is the most specific for oxytocin.

It is not surprising that the slopes of regression of lysine vasopressin and U.S.P. Standard are not parallel when assayed in the 0.5 mM/l. magnesium solution. Rather, it is surprising that the slopes of regression of more of the substances assayed do not show significant deviations from parallelism with the U.S.P. Standard. It is entirely possible that if more six-point or eight-point assays had been employed slight deviations from parallelism with the Standard would have been proved significant for many of these substances. Deviation from parallelism must be considerable in the usual four-point assay before its significance can be established.

Of the substances assayed valyl oxytocin is most potentiated by the presence of 0.5 mM/l. magnesium. Since this is an impure analogue which contains other substances used in its synthesis the author believes that these results should be interpreted with caution. Berde, Doepfner and Konzett found that the *in vitro* rat oxytocic potency of valyl oxytocin was 2.8 U/ml.[5] and that the *in vivo* rat oxytocic potency was approximately 10 U/ml. (24). The ratio of the second to the first is 3.6, which is equal to the ratio found by the author for the two *in vitro* assays (Table 2). This ratio *equality* is probably fortuitous, but it is possible that the reported differences between *in vitro* and *in vivo* potencies are largely attributable to the absence or near absence of magnesium from the bath solution used for the *in vitro* assay.

The solution of van Dyke and Hastings with elimination or modification of its magnesium concentration has been shown to be excellently adapted to the rat oxytocic assay. Its physiological and fixed pH, its near replication of an ultrafiltrate of mammalian plasma and its buffering capacity all make it logically preferable to the various modifications of Ringer-Locke solution. For assays of most neurohypophysial preparations a magnesium-free solution provides the most suitable and specific assay for oxytocin. Use of the same unknown preparation assayed against a suitable standard with and without magnesium in the bath solution provides a useful qualitative analytical tool, for oxytocin is the only octapeptide studied whose potency is not increased by the presence of magnesium in the bath. Qualitative analytical use has already been made of this pharmacological phenomenon (25).

REFERENCES

1. DALE, H. H.: *J. Physiol.* **34**: 163. 1906.
2. DALE, H. H. AND P. P. LAIDLAW: *J. Pharmacol.* **4**: 75. 1913.

[5] The composition of the bath solution used in this investigation is not known to the author.

3. KOCHMANN, M.: *Hoppe-Sayler's Zeit. Schr. f. Physiol. Chem.* **115:** 305. 1921.
4. BURN, J. AND H. H. DALE: *Spec. Rec. Ser. Med. Res. Coun. Lond.* **69:** 1. 1922.
5. VAN DYKE, H. B. AND A. B. HASTINGS: *Am. J. Physiol.* **83:** 563. 1928.
6. MILLER, C. P. AND A. B. HASTINGS: *Proc. Soc. Exper. Biol. & Med.* **27:** 748. 1930.
7. GENELL, S.: *Skand. Arch. Physiol.* **80:** 124. 1938.
8. FRASER, A. M.: *J. Pharmacol.* **66:** 85. 1939.
9. STEWART, G. A.: *J. Pharm. & Pharmacol.* **1:** 436. 1949.
10. The British Pharmacopoeia, 1948. London, Constable & Co., p. 813, 1948.
11. FITZPATRICK, R. J.: *J. Pharm. & Pharmacol.* **8:** 403. 1956.
12. BEAUVILLAIN, A.: *Comp. Rend. Soc. Biol.* **137:** 284. 1943.
13. HOLTON, P.: *Brit. J. Pharmacol.* **3:** 328. 1948.
14. SCHILD, H. O.: *J. Physiol.* **101:** 115. 1942.
15. BURN, J. H., D. J. FINNEY AND L. G. GOODWIN: *Biological Standardization*, (2nd Ed.) New York, London, Oxford University Press. 1950.
16. PIERCE, J. G., S. GORDON AND V. DU VIGNEAUD: *J. Biol. Chem.* **199:** 929. 1952.
17. TURNER, R. A., J. G. PIERCE AND V. DU VIGNEAUD: *J. Biol. Chem.* **191:** 21. 1951.
18. POPENOE, E. A., H. C. LAWLER AND V. DU VIGNEAUD: *J. Am. Chem. Soc.* **74:** 3713. 1952.
19. VAN DYKE, H. B., S. L. ENGEL AND K. ADAMSONS, JR.: *Proc. Soc. Exper. Biol. & Med.* **91:** 484. 1956.
20. VAN DYKE, H. B., R. A. MUNSICK AND W. H. SAWYER: *Fed. Proc.* **18:** 163. 1959.
21. POPENOE, E. A., J. G. PIERCE, V. DU VIGNEAUD AND H. B. VAN DYKE: *Proc. Soc. Exper. Biol. & Med.* **81:** 506. 1952.
22. VAN DYKE, H. B.: Personal communication.
23. FINNEY, D. J.: *Statistical Method in Biological Assay*. New York, Hafner Publishing Company, 1952.
24. BERDE, B., W. DOEPFNER AND H. KONZETT: *Brit. J. Pharmacol.* **12:** 209. 1957.
25. MUNSICK, R. A., W. H. SAWYER AND H. B. VAN DYKE: *Fed. Proc.* **18:** 110. 1959.

THE ISOLATION AND IDENTIFICATION OF SOME ADRENOCORTICOSTEROIDS RELEASED BY RAT ADRENAL TISSUE INCUBATED *IN VITRO*[1]

FERNAND G. PÉRON

With the Technical Assistance of WILLIAM F. ROBIDOUX

The Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts

ABSTRACT

A lipid extract of the pooled preincubation media of incubated rat adrenal sections or of rat adrenal tissue homogenates incubated *in vitro* was chromatographed on paper chromatograms. Four distinct zones absorbing ultraviolet light were isolated when the chromatograms were run in the toluene-propylene glycol system. These zones were designated as X_1, aldosterone, X_2 and corticosterone. Aldosterone as the 21-monoacetate and free corticosterone were isolated in the crystalline state and identified by infrared spectroscopy and other criteria. The two compounds, X_1 and X_2, were not identified although X_2 was crystallized and several of its physical and chemical characteristics noted. One of these was its ability to react with the sulfuric acid phenylhydrazine reagent to yield a chromogen absorbing light at 400–410 mμ. The importance of X_2 was discussed from the point of view of its possible physiological significance.

NUMEROUS workers (1–16) have shown that corticosterone is the main steroid produced by the rat adrenal gland *in vivo* and *in vitro*. Therefore, the rat adrenal gland has generally been classified as a corticosterone producer. On the basis of paper chromatographic and bioassay procedures, aldosterone has been shown to be one of the substances elaborated in minor amounts by the rat adrenal gland (3, 17). Another, a substance of unknown structure has been shown to yield a chromogenic material (6, 16, 18, 27, 28) after its reaction with the phenylhydrazine sulfuric acid reagent of Silber and Porter (19). In studies dealing with the possible mechanism(s) which would explain the mode of action of the adrenocorticotropic hormone (4, 5, 20, 21), we have also consistently detected on paper chromatograms several ultraviolet absorbing substances, in addition to corticosterone. some of these substances have properties similar to those reported by other investigators (1, 3, 7, 8, 16, 18, 28). However the amount of materials isolated was always too small to permit positive identification by more rigorous criteria besides paper chromatography or bioassay only. It is with the purpose of isolating much larger amounts of the ultraviolet ab-

Received October 5, 1959.

[1] This work was supported in part by research grants from the National Science Foundation (No. G-1448), the United States Public Health Service Grant A-2672 and the United States Atomic Energy Commission, Contract No. AT(30-1)-916.

sorbing materials that this work was carried out. It has been found that one of these ultraviolet substances is aldosterone and that another, a compound designated as X_2 (see text), is a Porter Silber chromogenic substance having characteristic physical and chemical properties. These differ from 17α-hydroxy-21-oxopregn-4-ene-3,20-dione a compound which is thought as being similar to it (18). A third compound absorbing ultraviolet light and isolated in the crystalline state was found to be corticosterone.

METHODS

General. In a group of studies (4, 5, 20, 21) wherein a great number of *in vitro* incubations with rat adrenal sections were carried out, the preincubation media in these experiments were pooled and stored in the frozen state. Since the number and the location of the steroids visualized on paper chromatograms of dichloromethane extracts of this medium do not differ from those found in extracts of the final incubation medium (4, 5, 20), the pooled preincubation media served as one source for obtaining the steroids elaborated by the rat adrenal gland. Approximately four liters of this preincubation medium was collected and extracted as described below. In order to supplement the amount of steroids obtained in this manner the adrenals of 800 Sprague-Dawley rats[2] were homogenized, activated by freezing (23) and incubated by methods described for rat adrenal sections or homogenates (4, 23). These homogenates which produce the same steroids as adrenal sections incubated in the same manner, were pooled, stored in the frozen state and then extracted.

Extraction of Steroids. The steroids in the pooled preincubation medium and in the pooled homogenates were obtained by extracting the aqueous phases twice with two times their volume of dichloromethane. The dichloromethane was next evaporated under vacuum and the resulting residue partitioned between 200 ml. of 80% methanol-water (80/20, v/v) and 50 ml. petroleum ether (boiling point 30–60° C). A second partition was carried out by using a fresh portion of petroleum ether after which the pooled petroleum ether phases were extracted twice with 20 ml. of 80% methanol-water. The pooled methanolic phases were brought to dryness under vacuum and the dry residue was dissolved in 4.0 ml. of dichloromethane and then divided into four 1.0 ml. aliquots.

Paper Chromatography. After running the four dichloromethane aliquots respectively on 4, 15 cm-wide paper chromatograms in the toluene-propylene glycol system (24) for 40 hours at 27° C the latter were dried and examined for substances absorbing ultraviolet light. A narrow test strip was also cut along the edge of each chromatogram in order to observe any substances which would react with the blue tetrazolium (1) or the Zimmermann reagents (25). In no instance was there found substances which reacted with the Zimmermann reagent. The corresponding zones on each of the chromatograms which were delineated by ultraviolet light and the blue tetrazolium reagent were cut out and eluted with large volumes of methanol. The methanol was next evaporated under vacuum and each eluate partitioned between dichloromethane and water. Most of the extraneous nonsteroidal substances which were extracted by the methanol from the paper chromatograms were soluble in the water phase of the partition system and therefore removed. Rechromatographing, reelution and repartitioning of the individual zones was carried out exactly as described above.

Column Chromatography. After removal of the dichloromethane from the eluates of

[2] The author wishes to thank Dr. Eugenia Rosemberg of the Worcester State Hospital, Mass., for making available a plentiful supply of rat adrenal glands and a sample of standard dl-aldosterone 21-monoacetate. He is also indebted to her for carrying out the bioassay work on the material suspected as being aldosterone-21-monoacetate.

the second paper chromatograms the dry residue which remained was redissolved in dichloromethane and transferred quantitatively to 1.0 gm. of Celite (Celite 545 Johns-Manville). Following the addition of small amounts of 16-^{3}H corticosterone (0.30 μg. = 0.90 μC) or of 16-^{3}H corticosterone 21-monoacetate (0.06 μg. = 0.18 μC) respectively to the Celite the latter was dried thoroughly under high vacuum. To the dry Celite containing the paper chromatogram eluates of free X_2 and the 16-^{3}H corticosterone was added 0.50 ml. of the stationary phase of a modified Bush B3 system (26) (Skellysolve C (boiling point 93–100° C):benzene:water:methanol; 3:17:1:1 volumes respectively) and mixed. The Celite was next transferred as a slurry in about 5.0 ml. of the mobile phase and packed on top of a Celite column prepared by mixing 28.0 gm. of Celite with 14.0 ml. of stationary phase as described by Ayres *et al.* (26). The final height of the column was 60.0 cm. and had a diameter of 1 cm. (column 1).

Similarly the acetylated product of X_2 was chromatographed on a Celite column. In this instance 16-^{3}H corticosterone 21-monoacetate instead of 16-^{3}H corticosterone was added to the 1.0 gm. of Celite (cf. above). The stationary and mobile phase used to prepare and elute the column was made up of Skellysolve C:benzene:water:methanol; 3:1:4:1 respectively (column 2).

The colums were eluted in fractions of 5.0 ml. and a small portion of each analyzed for radioactivity in a TRI-CARB scintillating counter, Model 314-DC at an efficiency level of about 16%. Since free and acetylated X_2 had approximately the same paper chromatographic mobilities as corticosterone and its 21-monoacetate derivative respectively, the localization of the radioactivity in the different fractions served to indicate in what fraction X_2 or its monoacetate would be eluted from the columns. The distribution of the radioactivity in the fractions gave a measure of the efficiency of the columns which is assessed in terms of theoretical plates[3] (39).

RESULTS

Initial Paper Chromatograms. Four distinct zones which absorbed ultraviolet light were observed on the four paper chromatograms run in the toluene-propylene glycol system for 40 hours. This finding is in agreement with those reported by other investigators (2, 8, 27, 29). The individual zones on each of the chromatograms were designated, in order of decreasing polarity, as X_1, aldosterone, X_2 and corticosterone zones respectively. Although all of the zones absorbed ultraviolet light only the aldosterone and the corticosterone zones reacted with the blue tetrazolium reagent. The X_1 zone occupied a position which cortisol usually occupies when run in the same system (about 3–5 cm. from the starting line) while the corticosterone zone was observed near the lower edge of the chromatogram. The aldosterone zone had the same chromatographic mobility as cortisone and X_2 moved more slowly than corticosterone. The individual zones, as designated above, were pooled and eluted with methanol.

Identification of the Pooled Zones. X_1 zone—This material after elution and desiccation was rerun in the toluene-propylene glycol system for a period of 64 hours on a 3 cm-wide chromatogram. This permitted the sub-

[3] The author also wishes to express his gratitude to Dr. J. Tait and Mrs. S. Tait for their help in the preparation of the Celite columns, for the tritriated corticosterone and their overall genuine interest and help in the problem of identification of steroid X_2.

stance to move about one quarter to half-way down the paper chromatogram. After drying, the chromatogram was examined under ultraviolet light. One very dark zone was observed which gave a very slight reducing reaction with the blue tetrazolium reagent. The rate of reduction of the reagent was slow and a reddish-blue color appeared only after a few minutes as compared to an almost instantaneous reaction with cortisol or corticosterone. The Zimmermann reaction (25) was negative when applied to a test strip cut from the main chromatogram. The zone also gave positive dinitrophenylhydrazine (25) and soda fluorescence reactions (9). Unfortunately, the very small amount of material which was eluted from the paper chromatogram precluded any attempts directed at its identification by classical means. For this reason, the material was stored in a desiccator until more can be obtained.

Aldosterone Zone. The zones corresponding to aldosterone in mobility were cut out and eluted. After pooling of the eluates these were rerun on an 8 cm-wide chromatogram for 64 hours in the toluene-propylene glycol system. After drying, the chromatogram was examined in the usual manner. Two very small zones absorbing ultraviolet light were observed on the chromatogram in addition to the large zone corresponding in position to that of standard aldosterone. These minor zones corresponded in position to those of compound X_2 and corticosterone (cf. below). After elution of the aldosterone zone with methanol and its evaporation the viscous residue was partitioned between 100 ml. dichloromethane and 20 ml. of water as described and for the reasons noted in the Methods section. The partition was repeated using a fresh 20 ml. portion of water and the water phases discarded. After removal of the dichloromethane under vacuum, the residue was transferred to a 3 ml. conical tube and attempts were made to crystallize the yellowish semi-solid material. Following the failure to crystallize the substance, approximately $\frac{1}{4}$ of the total amount was mixed with potassium bromide (KBr) and then its infrared spectrum run (KBr prism (31)). A comparison of the infrared curve of an authentic sample of aldosterone (L-552-098-0-3) obtained from Dr. Edward A. Ham of Merck & Co. Inc., Rahway, New Jersey and run in the same manner, demonstrated that the curves were identical in all respects. A small amount of the isolated aldosterone when chromatographed on paper in the benzene formamide system (24) singly or admixed with authentic aldosterone had exactly the same mobility as the authentic compound. On the other hand, two clearly delineated spots which absorbed ultraviolet light and reduced the blue tetrazolium reagent were seen when cortisone was mixed with the isolated substance.

Following acetylation of the remaining isolated aldosterone according to the method of Mattox *et al.* (32), crystallization of the resulting acetate (21-monoacetate) was obtained. The acetate in the form of thick rhombic plates was isolated from an acetone-petroleum ether mixture (32). The

following were some of its physical and chemical characteristics: (a) Melting point 193–198° C (uncorrected) reported 193–195° C (32) and 198–199° C (33), (b) showed one absorption peak in concentrated H_2SO_4 at 288 mμ (room temperature 2 hours) while it showed additional peaks at 247, 342 and 460 mμ if the reaction was carried out at 90° C for 1 hr. This heating effect has been observed with free aldosterone isolated from ox adrenals (33), (c) had an ultraviolet absorption maximum in methanol at 240–241 mμ, (d) had an infrared spectrum (KBr pellet) which was identical to a sample of standard dl-aldosterone monoacetate[2] in all regions of the spectrum (400–1800 cm.$^{-1}$) except in the position of some bands in the fingerprint region. Although the latter were absolutely identical in all respects (height and width: with those obtained with the dl-aldosterone-21-monoacetate, there was some shifting of these bands to longer wavelengths. The author is unaware of any explanation for this effect although it is possible that the racemic and optically active material did not crystallize in the same lattice-type. This would give rise to considerable deviation in the infrared absorption spectrum especially in the fingerprint region.[4] Finally, (e) the isolated material had twice the biological activity of an equal amount of dl-aldosterone-21-monoacetate.

X_2 zone—The pooled eluates of zone X_2 obtained from the original chromatograms were rerun in the toluene-propylene glycol system for 40 hr. on two 15 cm-wide chromatograms. Preliminary purification of the eluted X_2 zones was carried out as for the aldosterone zone. The amount of substance absorbing ultraviolet light at 240 mμ in the resulting dry residue was found to be approximately 5.0 mg. (based on the supposition that its molecular weight and extinction coefficient were equal to that of corticosterone). Prior to chromatography of Celite column 1, five μg. amounts of the X_2 material were chromatographed singly and admixed with 5 μg. of corticosterone in the Bush B_5 system (9). Examination of the developed chromatogram in ultraviolet light showed that by far the greater amount of X_2 was made up of a more polar substance (Rf 0.33) than corticosterone (Rf 0.44). One minor constituent was present in the X_2 zone which moved much more rapidly than corticosterone (Rf 0.75). All spots gave a good soda fluorescence (9) while only the corticosterone spot gave a strong and almost immediate blue tetrazolium reaction. A faint blue tetrazolium reaction was given with the main X_2 spot (Rf 0.33) about 5–10 minutes after spraying the chromatogram with the reagent. In order to purify compound X_2 further it was chromatographed on column 1 as described in Methods. Following the collection of 63 fractions the radioactivity from the 16-^{3}H corticosterone was found in

[4] I would like to thank Dr. A. W. Wettstein of Ciba Limited, Switzerland, and Dr. R. Norman Jones of the National Research Council, Canada, for their interest in attempting to clarify and interpret the infrared spectrum of the isolated aldosterone-21-monoacetate.

fractions 9–13 being concentrated in fractions 11 and 12. From the distribution of radioactivity, the theoretical plate value of the column was assessed as geing of the order of 650 (39). By running 0.02 ml. aliquots of fractions 5–30 in the Bush B_5 system (9) most of compound X_2 was found in fractions 12 and 13 of the column eluate. In contrast to the original X_2 which was applied to column 1 the latter fractions were found to contain but one substance corresponding in position to the main constituent (Rf 0.33) found on the original Bush B_5 paper chromatogram. A white amorphous material was obtained from the combined fractions 12 and 13 (2.29 mg. by weight). The latter was dissolved in 1.0 ml. of ethyl acetate after the addition of 3 drops of methanol. The tube was allowed to stand at room temperature until crystals appeared and then placed in the cold. A total of 1.02 mg. of large rhombic plates or sheaths (Fig. 1) was harvested. After drying, the crystals had the following physical and chemical characteristics; (a) melting point, soft at 163° C, melted at 168–171° C (uncorrected). A second crop of crystals which had an identical infrared spectrum as that melting at 168–171° C melted at 180–183° C, (b) the crystals melting at 163° C had a

$$\lambda_{max.}^{MeOH} = 241 \text{ m}\mu \text{ and an } E_{1 \text{ cm.}}^{1\%} = 482$$

(c) no Zimmermann reaction was given by 15 or 30 μg of X_2 using the Holtorff and Koch procedure (35), (d) 5 μg. of the crystals gave a very faint reaction on paper with blue tetrazolium but did not reduce this reagent when the reaction was carried out in solution (20), (e) whereas 5 μg of corticosterone or cortisol on a 1 cm.^{2} area of paper reduced ammoniacal silver nitrate (36) quickly (1 minute), and 2 μg of 17α-hydroxy-21-oxopregn-4-ene 3,20-dione[5] immediately, 10–20 μg amounts of the crystals failed to reduce this reagent. However, 18-oxoallopregnane failed to reduce the silver nitrate reagent at low concentrations, (f) they failed to give rise to fluorescence in sulfuric acid (12) (g) gave one absorption peak in concentrated sulfuric acid (290–291 mμ) at room temperature without the appearance of additional peaks when the sulfuric acid solution was heated at 90° C for 1 hour (33), (h) behaved as a neutral compound when 50 μg of the crystals were partitioned between 2.0 ml. dichloromethane and 1.0 ml. 10% sodium carbonate, (i) gave the infrared spectrum seen in Figure 2, (j) gave a reaction with the phenylhydrazine sulfuric acid reagent of Porter and Silber (19). No reaction was given with the sulfuric acid alone. The plot of the optical density versus hours after addition of the Porter Silber reagent was linear from 3 hours to 24 hours at room temperature for X_2 and much less steep than at 1 or 2 hours. The optical density values at 400–410 mμ at the

[5] 17α-Hydroxy-21-oxopregn-4-ene-3,20-dione and 18-oxoallopregnane were obtained froms Drs. J. Tait and N. McNiven respectively of the Worcester Foundation.

FIG. 1. Crystals of Compound X_2 melting point 168–171° C. Photomicrograph of the crystals taken at 40 times magnification.

end of 24 hours for X_2 were approximately one half those obtained with equal amounts of cortisone. However, if the reaction was allowed to continue for 64 hours the final optical densities for the cortisone and X_2 were almost identical at the two concentrations used (4 and 8 μg).

Although column 1 failed to separate 16-^{3}H corticosterone from X_2 as was the case with the original impure X_2, 5 μg. of the crystals of X_2 (melting point 168–171° C) were clearly separated on a Bush B_5 paper chromatogram from 5 μg. of corticosterone. As only one spot was observed on the developed chromatogram of X_2 it can be concluded that it was not contaminated with the impurity (Rf .75) which was found in the original substance.

Approximately 400 μg. of crystalline X_2 were acetylated (37) and the acetylated product chromatographed on Celite column 2. Elution of the column, localization of the radioactivity and of the material absorbing ultraviolet light were carried out as for column 1. The radioactivity was found in fractions 37–44 and the ultraviolet absorbing material was asso-

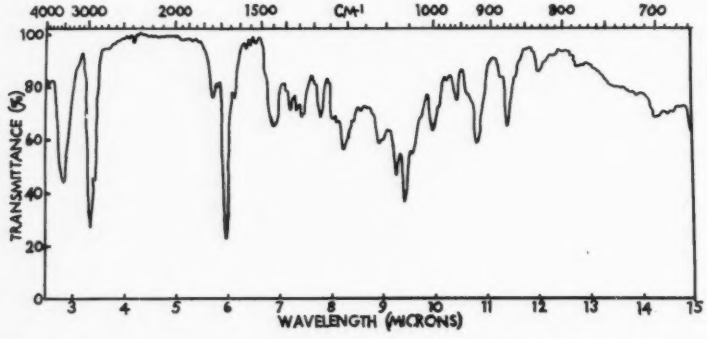


FIG. 2. Tracing of the infrared spectrum of compound X_2 run in KBr (31) on the Perkin-Elmer infracord spectrophotometer.

ciated with fractions 37–41. Fractions 38 and 39 contained most of the material absorbing ultraviolet light. The calculated theoretical plates of this column was found to be 450. One hundred μg. of X_2 acetate were obtained from pooled fractions 38 and 39. A comparison of the running rates of 5 μg. of X_2 acetate and corticosterone acetate in the Bush B_3 system showed that the former had the same or a slightly greater mobility. The infrared spectrum (KBr prism) of the remainder of X_2 acetate was such as to render its interpretation equivocal. However, since no absorption band in the 3600 cm^{-1} region of the spectrum was observed it can be concluded that no free hydroxyl groups were present on the compound after its acetylation.

Corticosterone zone. The pooled corticosterone zone eluates obtained from the first chromatograms were rerun in the toluene propylene glycol system on four 15 cm-wide paper chromatograms for 30 hours. The corticosterone zones were eluted in the usual manner. The partition of the eluate between 300 ml. of dichloromethane and 50 ml. of water was next carried out as previously described for aldosterone and X_2. This partition was repeated twice more with fresh 50 ml. portions of water. After evaporation of the dichloromethane the white semi-crystalline residue remaining was dissolved in a small volume of ethyl acetate and allowed to crystallize at room temperature. Crystals in the form of rods were obtained (about 2.0 mg.) which melted at 176–180° C (uncorrected). Admixture of this material with an authentic sample of corticosterone (melting point 178–182° C) melted at 173–177° C. The sulfuric acid chromogen curve (38) of this compound was identical with that of standard corticosterone. No evidence was obtained that epicorticosterone has been produced by the rat adrenal gland (27). The infrared spectrum (KBr pellet) of this substance was identical in all respects with that of authentic corticosterone run in the same manner.

DISCUSSION AND CONCLUSION

The isolation of aldosterone as the 21-monoacetate in the crystalline state constitutes the first time that this substance has been isolated in sufficient quantities from the rat adrenal gland to make its identification possible by rigid criteria rather than paper chromatography and bioassay only. Although it has been reported that the aldosterone isolated from chromatograms run in the toluene propylene glycol system for 40 hours was contaminated with cortisone (2), this fact could not be substantiated in the present study. The infrared data served to support this conclusion since identical spectra were obtained for the isolated substance and authentic aldosterone. On the basis of this evidence it is highly probable therefore that those zones formerly designated as EAL in other studies (4, 5) should have been denoted as aldosterone zones.

Of great interest in this study was the isolation of compound X_2. The

present and reported findings showing that this substance is produced by the rat adrenal gland incubated *in vitro* (8, 18, 27, 34), and its isolation on paper chromatograms from lipid extracts of rat blood (16) indicate that this substance may be secreted into the blood stream by the adrenal glands. The fact that the addition of adrenocorticotropin to rat adrenal glands incubated *in vitro* increased the production of the phenyl hydrazine reacting material markedly (29) also indicates that its synthesis and release from the rat adrenal gland may be controlled by the amount of adrenocorticotropin circulating in the blood. The value of assessing a function to the presence of this substance in the blood may be important on the basis of the data reported by other investigators (16, 40–42) suggesting that a similar substance(s) may also be present in other animal species. The question as to whether this substance has any physiological significance is a matter of conjecture at the present moment. However, the fact that it possesses an α,β-unsaturated 3-ketone grouping, one of the necessary attributes of all naturally occurring adrenocorticosteroids having a high degree of physiological activity, and is produced by the rat adrenal gland are reason enough to test this substance by bioassay procedures. The results of these experiments will be the subject of a future publication.

Because of the odd property of X_2 to react with the sulfuric phenylhydrazine reagent the elucidation of its structure was attempted. On the basis of the chemical and other evidence which has been obtained the position of the hydroxyl group(s) cannot be determined nor can it be said unequivocally that X_2 has a side chain at the C-17 position of the steroid nucleus. However, the results obtained from incubations carried out with rat adrenal glands when 21-^{14}C-deoxycorticosterone and progesterone respectively were used as substrates (2) indicated that in all probability X_2 has a C-21 side chain. This is deduced from the fact that the X_2 zones in these incubations were found radioactive and must have originated from the added precursors. The infrared spectrum of the free compound X_2 is interesting since it (Fig. 2) shows no carbonyl absorption band for a hexacyclic or side chain ketone group (1710–1720 cm^{-1}). The very small peak seen at this wave length (1720 cm^{-1}) is believed to be an artifact since the infrared spectrum of the same KBr prism when run on the Beckman Model IR-4 infrared spectrophotometer gave no indication that this band was present. Therefore, the infrared evidence does not indicate a C-20 ketone in its structure. The intensity of the band at 1745 cm^{-1} is such that it is much too small to attribute this peak to an unhindred pentacyclic ketone group. The fact that X_2 does not react with the Zimmermann reagent would also preclude the presence of a C=O group in ring D. On the other hand, there is the possibility that the 1745 cm^{-1} band may represent an aldehyde group. Some aldehydes are known to absorb infrared energy in the neighborhood of 5.75–5.81 mμ. (1738–1720 cm^{-1}). However, the intensity of this band is such that it precludes it as being due to a free

aldehyde group. In addition there is only a suggestion of an absorption band in the area where free aldehydes usually have a typical absorption band (2800–2700 cm^{-1}). The Beckman IR-4 spectrophotometer in spite of its high resolving power did not produce a better resolution of the bands in this region of the spectrum than the Perkin-Elmer infracord spectrophotometer (figure 2).

Although two compounds besides corticosteroids having the dihydroxy acetone side chain are known to react (43) with the sulfuric acid phenylhydrazine reagent of Porter and Silber to give characteristic chromogens, compound X_2 could not have the same structure at least with respect to C-21 side chain since the former compounds readily reduce ammoniacal silver nitrate. X_2 gave a negative silver nitrate reaction. By virtue of the reactions given by Δ^4-pregnene-20,20,21-triol 18 acid lactone-3-one and aldosterone 18–20 hemiketal as reported by Neher and Wettstein (44) these two compounds may be eliminated as possibilities. It is conjectured also that their paper chromatographic mobilities would be much less in the toluene-propylene glycol system than X_2. The compound Δ^4-pregnen-16α-ol-3,20-dione, recently isolated by the Ciba group from ox adrenal glands (4, 5) can also be eliminated as a possibility on the basis of its mobility on paper chromatograms. At this time, therefore, none of the adrenal steroids of known structure appear to be similar to X_2 and it is obvious that additional information is required before its structure can be elucidated.

The well known fact that corticosterone is the main steroid produced by the rat adrenal gland has been well substantiated in this investigation. A relatively large amount of this substance was obtained in contrast to the minute amounts of aldosterone and X_1 and the small amounts of X_2. Because of this fact no difficulty was experienced in its isolation and absolute identification.

The minute amount of X_1 which was isolated will be the subject of further investigations. Although its mobility was almost the same as that of cortisol in the toluene-propylene glycol system, its failure to react with the blue tetrazolium reagent indicates that this substance is not cortisol. This finding does not substantiate former claims that cortisol was produced by the rat adrenal gland (2, 20) but confirms the evidence obtained by other investigators using paper chromatographic (28–30) and fluorescent techniques (12–15), that the rat is not a cortisol producer. Since X_2 was found to have almost the same mobility as 17α-hydroxy-11-deoxycorticosterone in the toluene-propylene glycol system and since X_1 = cortisol in mobility in the same system, by analogy to compound S and cortisol the introduction of one more hydroxy group in X_2 would probably result in a compound which would have the same mobility on paper chromatograms as X_1. This, of course, is based on the supposition that X_1, apart from this additional hydroxyl group, has the same structure as X_2. Evi-

dence substantiating this suggestion was presented recently by Birmingham (18). Eluates of zone X_1 gave a positive reaction on paper with a sulfuric acid phenylhydrazine reagent (18) in addition to giving a retarded blue tetrazolium reaction. It did not reduce ammoniacal silver nitrate. Thus X_1 may be a hydroxylated form of X_2.

REFERENCES

1. Elliott, F. H. and A. H. Schally: *Canad. J. Biochem. & Phys.* **33**: 174. 1955.
2. Heard, R. D. H., R. Jacobs, V. O'Donnell, F. G. Péron, J. C. Saffran, S. S. Solomon, L. M. Thompson, H. Willoughby and C. H. Yates: *Recent Progr. in Hormone Res.* **9**: 383. 1954.
3. Giroud, C. J. P., M. Saffran, A. V. Schally, J. Stachenko and E. H. Venning: *Proc. Soc. Exper. Biol. & Med.* **92**: 855. 1956.
4. Koritz, S. B. and F. G. Péron: *J. Biol. Chem.* **230**: 343. 1958.
5. Péron, F. G. and S. B. Koritz, *J. Biol. Chem.* **233**: 256. 1958.
6. Birmingham, M. K. and E. Kurlents: *Endocrinology* **62**: 47. 1958.
7. Hoffman, F. G. and C. Davidson: *Endocrinology* **54**: 580. 1954.
8. McKerns, K. W., B. Coulomb, E. Kaleita and E. C. DeRenzo: *Endocrinology* **63**: 709. 1958.
9. Bush, I. E.: *Biochem. J.* **50**: 370. 1952.
10. Bush, I. E.: *J. Endocrinol.* **9**: 95. 1953.
11. Holzbauer, M. and M. Vogt: *J. Physiol.* **138**: 449. 1957.
12. Silber, R. H., R. D. Busch and R. Oslapas: *Clin. Chem.* **4**: 278. 1958.
13. Guillemin, R., G. W. Clayton, J. D. Smith and H. S. Lipscomb: *Endocrinology* **64**: 349. 1958.
14. Péron, F. G. and R. I. Dorfman: *Endocrinology* **64**: 431. 1959.
15. Fortier, C.: *Proc. Soc. Exper. Biol. & Med.* **100**: 13. 1959.
16. Reif, A. E. and B. B. Longwell: *Endocrinology* **62**: 573. 1958.
17. Singer, B. and M. P. Stack-Dunne: *J. Endocrinol.* **12**: 130. 1950.
18. Birmingham, M. K.: *Nature* **184**: 67. 1959.
19. Silber, R. H. and C. C. Porter: *J. Biol. Chem.* **210**: 923. 1954.
20. Koritz, S. B., F. G. Péron and R. I. Dorfman: *J. Biol. Chem.* **226**: 643. 1959.
21. Haynes, R. C., S. B. Koritz and F. G. Péron: *J. Biol. Chem.* **234**: 1421. 1959.
22. Saffran, M. and M. Bayliss: *Endocrinology* **52**: 140. 1953.
23. Koritz, S. B. and F. G. Péron: *J. Biol. Chem.* In Press.
24. Burton, R. B., A. Zaffaroni and E. H. Keutmann: *J. Biol. Chem.* **188**: 763. 1951.
25. Savard, K.: *J. Biol. Chem.* **202**: 457. 1953.
26. Ayres, P. J., O. Garrod, S. A. Simpson and J. F. Tait: *Biochem. J.* **65**: 639. 1957.
27. Eisenstein, A. B.: *Proc. Soc. Exper. Biol. & Med.* **91**: 657. 1956.
28. Hoffman, F. G.: *Endocrinology* **60**: 382. 1957.
29. Birmingham, M. K. and E. Kurlents: *Canad. J. Biochem. & Physiol.* **37**: 510. 1959.
30. Vogt, M.: *J. Physiol.* **130**: 601. 1955.
31. Rosenkrantz, H.: in D. Glick (Editor), Methods of Biochemical Analysis, Vol. II, Interscience Publishers, Inc., New York, 1955, p. 1–56.
32. Mattox, V. R., H. L. Mason and A. Albert: *J. Biol. Chem.* **218**: 359. 1956.
33. Simpson, S. A., J. F. Tait, A. Wettstein, R. Neher, J. vonEuw, O. Schlindler and T. Reichstein: *Helv. Chim. Acta* **37**: 1163. 1954.
34. Bligh, E. G., R. D. H. Heard, V. J. O'Donnell, J. L. Webb, M. Saffran and E. Schonbaum: *Arch. Biochem. & Biophys.* **58**: 249. 1955.
35. Holtorff, A. F. and F. C. Koch: *J. Biol. Chem.* **135**: 377. 1940.

36. ZAFFARONI, A. AND R. B. KEUTMANN: *Science* **111:** 6. 1950.
37. ZAFFARONI, A. AND R. BURTON: *J. Biol. Chem.* **193:** 749. 1951.
38. ZAFFARONI, A.: *J. Am. Chem. Soc.* **72:** 3828. 1950.
39. TAIT, S. A. AND J. F. TAIT: Memoirs of the Society of Endocrinology—Cambridge University Press—In press.
40. MCDONALD, I. R. M. AND M. REICH: *J. Physiol.* **147:** 33. 1959.
41. DRYRENFURTH, I., S. SYBULSKI, V. NOTCHEV, J. C. BECK AND E. H. VENNING: *J. Clin. Endocrinol. & Metab.* **18:** 391. 1958.
42. TOUCHSTONE, J., D. Y. COOPER AND W. S. BLAKEMORE: *J. Clin. Endocrinol. & Metab.* **19:** 812. 1959.
43. SILBER, R. AND C. C. PORTER: *Methods of Biochemical Analysis.* Academic Press Inc., Vol. 4, p. 139. 1957.
44. NEHER, R. AND A. WETTSTEIN: *Helv. Chim. Acta* **39:** 2062. 1956.
45. WETTSTEIN, A., R. NEHER AND H. J. URECH: *Helv. Chim. Acta* **42:** 956. 1959.

ENDOCRINE EFFECTS OF METHYLENEDIANILINE IN THE RAT, RABBIT AND DOG

WILLIAM W. TULLNER

U. S. Department of Health, Education and Welfare, Public Health Service, National Cancer Institute, Endocrinology Branch, Bethesda, Maryland

ABSTRACT

Effects of methylenedianiline were investigated in the rat, the rabbit, and the dog. In the castrate female rat, this compound produced adrenal hypertrophy, enlargement of the uterus, and was goitrogenic. In the dog, methyleñedianiline effected a marked inhibition of adrenal 17-hydroxycorticoid secretion. In the immature female rabbit, this compound elicited progestational proliferation of the endometrium which was shown to be adrenal-dependent. Adrenalectomy as well as cortisone-treatment blocked the progestational effect of methylenedianiline. Inasmuch as these effects are also characteristic of amphenone, these studies provide evidence that the methyl ketone structure is not essential for the responses observed. However, methylenedianiline lacks the marked anesthetic effect of amphenone.

THE unique endocrine effects produced by amphenone (1, 2, 3) have led to further investigations of less complex, related compounds in order to determine structural requirements for specific endocrine alterations.

The following animal experiments demonstrate certain endocrine responses to 4,4′ methylenedianiline, a diaminodiphenyl compound which lacks the ketone and the methyl groups of amphenone (Fig. 1). Results described here indicate that many of the pharmacological effects of methylenedianiline are similar to those of amphenone including the inhibition of adrenocortical steroid secretion in the dog (3) although the marked anesthetic effect of the latter compound was no longer apparent.

MATERIALS AND METHODS

In each of the experiments described, methylenedianiline dihydrochloride was administered in aqueous solution.

In studies on the rat, Sprague-Dawley strain females, weighing 100–150 gm. were bilaterally ovariectomized. Treatment was begun 18 days later. The compound was given daily by gavage in 0.5 ml. solution at 150 mg./kg. to 200 mg./kg. Commercial stock diet was available *ad lib.* The animals were sacrificed by exposure to chloroform. Body and organ weights were recorded. Tissues were fixed in Bouin's solution and stained by routine hematoxylin and eosin.

In the dog studies, 15 adult mongrel males were used. A standard commercial ration was fed. Body weights ranged from 5–17 kg. The drug was administered by the intra-

Received October 22, 1959.

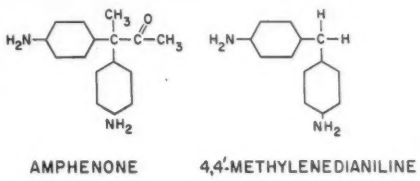


FIG. 1. A comparison of the structural formulas of 4,4'-methylenedianiline and amphenone.

venous route. Details of adrenal vein cannulation and 17-hydroxycorticoid determinations have been described previously (3).

In studies on the rabbit, progestational activity was determined by means of a modified Clauberg assay. Immature female New Zealand white rabbits (600–800 gm.) from a single colony were used. All drugs were injected subcutaneously. Methylenedianiline dihydrochloride was given in 1 ml. aqueous solution for each 100 mg. administered. Methods of treatment, surgical techniques, autopsy procedures and standards for interpretation of the progestational responses have been described (2). At autopsy adrenal weights were recorded to the nearest mg. on a Roller-Smith torsion balance.

In the tests for possible anesthetic properties of methylenedianiline a 2% aqueous solution of the drug was injected slowly into the marginal ear vein of the adult male New Zealand white rabbit.

RESULTS

Endocrine Effects in the Rat

In the castrate female rat, daily oral administration of methylenedianiline dihydrochloride at doses of 150 mg./kg. to 200 mg./kg., for periods of 5 to 14 days, resulted in adrenal, thyroid, and uterine hypertrophy. Table 1 shows results on a representative series of animals after 14 days of treatment. Although the mean body weight of the treated animals was 17% less than that of the untreated controls, the mean uterine, adrenal, and thyroid weights of the treated groups represented an increase of 71%, 57% and 92% respectively. Histologically, the zona fasciculata and zona reticularis of the adrenals from the treated animals showed extensive lipid accumulation. Thyroid follicles in these animals contained little or no colloid. The endometrium presented an atypical folliculoid response such as that observed following amphenone treatment (1).

Effects of Adrenocortical Secretion in the Dog

In a series of 15 dogs, acute intravenous administration of methylenedianiline dihydrochloride was followed by a prompt decrease in the secretion

TABLE 1. EFFECTS OF METHYLENEDIANILINE IN CASTRATE ♀ RAT

Treatment	No. of rats	Final body wt. (g.)	Uterine wt. (mg.)	Adrenal wt. (mg.)	Thyroid wt. (mg.)
Methylene-dianiline*	9	186 ± 26	104 ± 14	102 ± 17	25 ± 4
None	5	225 ± 10	61 ± 15	65 ± 5	13 ± 2

* Given as the dihydrochloride at 150 mg./kg. in 0.5 cc. H_2O daily orally for 14 days.

rate of 17-hydroxycorticoids. Doses ranging from 50 mg./kg. to 100 mg./kg. were given as 2.5% aqueous solutions. Results of representative experiments on two dogs are shown in Figure 2. A single injection of the drug produced a 7 to 8-fold decrease in 17-hydroxycorticoid output 5 minutes after administration. This decrease was followed by partial recovery of secretory capacity 60 minutes later. In experiments in which blood samples were collected two hours post-treatment, the 17-hydroxycorticoid content

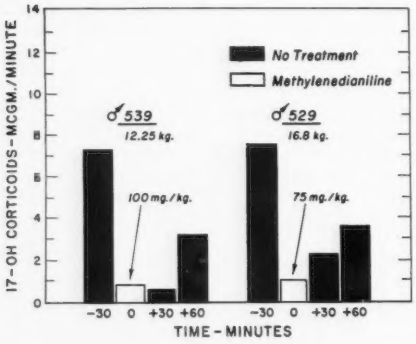


FIG. 2. Effect of methylenedianiline on 17-OH corticoid secretion in the dog.

had returned to pre-treatment levels. Following drug administration at these levels, there was a temporary rise in arterial blood pressure together with an increased blood flow from the lumbo-adrenal vein.

Effects in the Rabbit

In a series of experiments (Table 2) methylenedianiline and progesterone were compared with respect to endometrial and adrenal weight effects in the rabbit. In Series I, a total dose of 1 gm. of methylenedianiline was required to elicit a progestational response equivalent to that of 0.5 mg. of progesterone. This indicates an activity approximately 0.05% that of progesterone. The mean adrenal weight in the progesterone-treated group was 95 mg. while the mean of the group receiving methylenedianiline (1 gm.) increased to 169 mg. In order to eliminate possible endogenous sources of progesterone as a factor in the progestational response to methylenedianiline, additional series of animals were either ovariectomized or adrenalectomized and ovariectomized. Results in Table 2, Series II show that methylenedianiline as well as progesterone induced a progestational response in the absence of the ovaries. However, following combined ovariectomy and adrenalectomy (Series III, Table 2), methylenedianiline did not produce a progestational response. Progesterone, on the other hand, exerted its characteristic effect.

In another series (Series IV, Table 2), intact rabbits were treated with methylenedianiline, cortisone acetate, progesterone and combinations of each. When cortisone acetate (12.5 mg.) was administered together with

methylenedianiline (1 gm.), the progestational response was suppressed. The adrenals in this group had atrophied (mean = 64 mg.) whereas the adrenals of those animals on methylenedianiline alone were markedly hypertrophied (mean = 144 mg.). The mean adrenal weight of the progesterone-treated group was 92 mg. Cortisone acetate (12.5 mg.) alone did not affect the progestational response to progesterone.

A group of 3 rabbits which did not receive estrogen prior to treatment with methylenedianiline (1 gm.) failed to exhibit a progestational response.

Methylenedianiline was also tested for possible anesthetic effects in the rabbit. Doses of 100 mg./kg. were lethal in 2 rabbits and, at lower doses (66 mg./kg.), there was no evidence that methylenedianiline produced an anesthetic effect in a group of 4 rabbits.

TABLE 2. EFFECTS OF ALTERED ENDOCRINE STATUS ON THE PROGESTATIONAL RESPONSE TO METHYLENEDIANILINE

Series	No. of rabbits	Compound	Total dose (mg.)	Operative procedure	Progestational proliferation
I	4	Methylenedianiline	1000.0	None	+4, +4, +4, +4
	3	Methylenedianiline	250.0	None	+1, 0, +1
	4	Progesterone	.5	None	+4, +3, +4, +4
II	3	Methylenedianiline	1000.0	Ovariectomy	+4, +4, +4
	3	Progesterone	.5	Ovariectomy	+4, +4, +4
III	3	Methylenedianiline	1000.0	Adrenalectomy +Ovariectomy	0, 0, 0
	2	Progesterone	.5	Adrenalectomy +Ovariectomy	+4, +4
IV	3	Cortisone	12.5	None	0, 0, 0
	2	Methylenedianiline	1000.0	None	+4, +4
	2	Methylenedianiline +Cortisone	1000.0 12.5	None	0, 0
	2	Progesterone	.5	None	+4, +4
	3	Progesterone +Cortisone	.5 12.5	None	+4, +3, +3

† Ten μg. estradiol-17 beta administered in 0.25 ml. sesame oil solution daily for 6 days followed by 5 days treatment with test compounds. All compounds injected subcutaneously.

DISCUSSION

The pharmacological effects of methylenedianiline (diaminodiphenylmethane) described here are in many respects similar to those elicited by the diaminodiphenyl compound amphenone when the same species are treated under similar experimental conditions. In the rat, the pronounced goitrogenic effect observed after methylenedianiline treatment confirms the earlier report by Astwood *et al.* (4). A similar goitrogenic action has been reported for amphenone (1).

One of the most noteworthy differences between the two compounds was seen in the dog. Although both amphenone and methylenedianiline effect a marked inhibition in the secretion rate of adrenocortical steroids,

the consistent increase in arterial blood pressure following administration of the latter compound was in marked contrast to the temporary decrease in pressure usually seen after amphenone treatment. This blood pressure effect may be explained by the known anesthetic qualities of amphenone which when superimposed on the sodium pentobarbital anesthesia produce a fall in blood pressure. Methylenedianiline, on the other hand, has been shown to have no anesthetic action so that here the probable stimulus for the transient rise in arterial pressure was the physiological response to the increase in circulating fluid volume following the rapid intravenous injection of aqueous solutions (25 to 50 ml.) of the drug.

In the rabbit, the progestational response to methylenedianiline which was found to be mediated by the adrenal glands is similar to the adrenal progestational effect of amphenone except that the potency of the former compound is approximately 3 times that of amphenone.

It is apparent that methylenedianiline requires an estrogen-primed uterus in order to induce the adrenal-progestational response. Progesterone alone, however, can exert such a response at higher doses (5). Possible mechanisms for adrenal progestin secretion have been discussed in an earlier paper (2).

The findings reported here demonstrate that the methyl ketone structure is not essential for endocrine effects previously reported for amphenone.

Acknowledgment

The invaluable assistance of Mr. Donald L. Barber and Mr. Howard Erwin is gratefully acknowledged.

REFERENCES

1. Hertz, R., W. W. Tullner, J. A. Schricker, F. G. Dhyse and L. F. Hallman: *Recent Progress in Hormone Research* **11**: 119. 1958.
2. Tullner, W. W.: *Proc. Soc. Exper. Biol. and Med.* **98**: 157. 1958.
3. Tullner, W. W., M. M. Graff and R. Hertz- *Endocrinology* **58**: 802, 1956.
4. Astwood, E. B., A. Bissell and A. M. Hughes: *Endocrinology* **37**: 456. 1945.
5. Hisaw, F. L., R. O. Greep and H. L. Fevold: *Proc. Soc. Exper. Biol. and Med.* **36**: 840. 1937.

OVARIAN RESPONSE TO ADMINISTRATION OF HYPOPHYSEAL FOLLICLE-STIMULATING OR HUMAN CHORIONIC GONADOTROPHIC HORMONE IN ALLOXAN DIABETIC RATS[1]

A. M. LAWRENCE AND A. N. CONTOPOULOS[2]

Department of Anatomy, School of Medicine, University of California, San Francisco, and

Institute of Experimental Biology, University of California, Berkeley, California

ABSTRACT

Atrophy of the female rat reproductive system nineteen days after administration of a diabetogenic dose of alloxan was comparable to that reported in nineteen day post-hypophysectomized adult female rats. The administration of 80 μg. of FSH produced positive stimulation to follicular growth with the formation of small-medium follicles. No effect on the interstitial tissue was found at the highest administered FSH dose of 320 μg. The administration of 100 μg. HCG resulted in repair of interstitial tissue and at the highest dose (400 μg.) in development of large follicles and corpora lutea. It is suggested that the major factor accounting for impaired gonadal function is a defective or decreased release of hypophyseal gonadotrophic hormones.

REPRODUCTIVE dysfunction and associated alterations in the histological appearance of the gonads and accessory reproductive organs in cases of diabetes mellitus have been documented clinically (1, 2), and experimentally (3, 4, 5, 6). Recent pituitary bioassay studies have shown that the anterior pituitaries of alloxanized diabetic female rats contain gonadotrophic hormones in amounts comparable to those of control rats (7). The present study was undertaken in an effort to elucidate whether deficient ovarian response to gonadotrophic hormones could be a partial cause for the reproductive dysfunctions seen in the diabetic rat.

MATERIALS AND METHODS

Female rats of the Long-Evans strain were made diabetic with alloxan at 59–66 days of age (8). All animals were fasted approximately 72 hours prior to tail vein administration of 5% saline solution of alloxan, in a dose of 5 mg./100 gm. of body weight. Immediately following rapid injection of the drug, all animals were allowed access to food. Fourteen days after alloxan administration these animals received subcutaneous injections of saline solutions of follicle-stimulating[3] (FSH) or human chorionic gonado-

Received November 3, 1959.

[1] Supported in part by a grant from the U. S. Pbulic Health Service (RG-4965).

[2] The authors gratefully acknowledge the guidance and critical judgment extended by Doctors Miriam E. Simpson and Marjorie M. Nelson.

[3] FSH in powder form was obtained from the Institute of Experimental Biology and

trophic hormone[4] (HCG). Total doses of 80, 160 and 320 μg. of follicle-stimulating hormone and total doses of 100, 200 and 400 μg. of human chorionic gonadotrophic hormone were given in four daily injections. Animals were autopsied 24 hours after the last injection of hormone, nineteen days after alloxan administration. At the time of autopsy the ovaries and uteri were examined under a dissecting microscope, dissected out, weighed, and fixed in Bouin's fluid.

Rats were fed a standard laboratory diet[5] *ad libitum* and given free access to tap water. Twice a week the diet was supplemented with fresh lettuce. All rats were maintained at a temperature of 74° ± 1 F.

Non-fasted blood and urine glucose levels were determined by Nelson's modification of the Symogyi procedure (8). Only rats with glycosuria and persistent blood sugar levels above 400 mg.% for ten days prior to hormone injections were judged diabetic in this experiment.

Evaluation of the effect of hormone preparations on ovarian and uterine morphology and function was based upon the organ weights and the study of hematoxylin and eosin stained 8 μ secions of ovaries from treated and non-treated diabetic rats as well as on comparison of such gonads with those from normal non-diabetic rats of the same age. The dose of follicle-stimulating hormone which produced healthy small-medium follicles with beginning antrum formation was considered as the minimum effective dose. Beginning repair of ovarian interstitial tissue was judged by an increase in the vesicularity of the nuclear pattern, accompanied by an increase in the size, and depth of staining of these cells.

RESULTS

Untreated normal and Diabetic rats: At autopsy, nineteen days after alloxan administration, atrophy of the ovaries and accessory reproductive organs was grossly evident in the diabetic untreated rats (Table 1). The mean weight of ovaries from non-diabetic normal rats was 59 mg.; more than double that of ovaries from non-treated diabetic rats (21 mg.). Histologic examination of the untreated diabetic rat ovary revealed small-healthy and occasional small-medium atretic follicles. The ovarian interstitial cells were atrophic, with pyknotic nuclei and scanty cytoplasm. The normal control ovaries showed small, medium and large follicles, corpora lutea were present, and there was normal, healthy appearing interstitial

was prepared for sheep pituitaries according to the method of Jensen *et al.* (11) This preparation has been standardized in immature hypophysectomized female rats for follicle-stimulating activity. The minimum effective dose of 40 μg., or one rat unit (12), injected subcutaneously over four days, was found to elicit a minimal response of follicular growth characterized by healthy follicles with just beginning antrum formation.

[4] HCG was obtained in powder form as a commercial preparation supplied by Winthrop. This hormone has been re-standardized by its ability to cause repair of ovarian interstitial tissue in immature hypophysectomized rats of the Long-Evans strain. A total dose of 200 μg. caused partial repair of ovarian interstitial tissue in the test animal; 400 μg. (1RU) produced full repair of interstitial tissue in these rats.

[5] All animals were maintained on a modified McCollum Diet I which consists of ground whole wheat 67.5%, casein 15%, skim milk powder 7.5%, sodium chloride 0.75%, calcium carbonate 1.5%, melted fat 6.75%, fish oil (Vit. A and D concentrate) 1%, KI solution added to provide 1 μg. of I per gm. of diet.

tissue. The average uterine weight of normal non-diabetic rats was 357 mg. as opposed to the 64 mg. average uterine weight in non-treated diabetic rats (Table 1). It is interesting that the degree of atrophy seen in the non-treated diabetic rat, nineteen days after alloxan administration, is comparable in magnitude to involution of the reproductive system in nineteen-day post-operative hypophysectomized rats of the same age and strain (9).

FSH-treated Diabetic rats: A total dose of 80 or 160 μg. FSH did not appear to augment ovarian weights in diabetic rats (Table 1). On histological examination, however, the appearance of the ovaries from this group did disclose positive stimulation of follicular growth. Healthy small and small-medium follicles were present, interspersed in the sub-normal interstitial

TABLE 1. OVARIAN AND UTERINE RESPONSE OF ALLOXAN DIABETIC RATS TREATED WITH FOLLICLE-STIMULATING HORMONE (FSH) OR HUMAN CHORIONIC GONADOTROPHIC (HCG)

Group	Total dose, μg.	No. of rats tested	Uterus weight, mg.	Ovaries		
				Weight, mg.	Follicles†	Interstitial tissue
Normal Rats	0	3	357 ± 54*	59 ± 4	sm, ml, & CL	Normal
Diabetic Non-Treated	0	3	64 ± 3	21 ± 2	s to atretic sm	Deficient
Diabetic FSH	80	3	87 ± 10	34 ± 5	s, sm	Deficient
	160	3	91 ± 10	33 ± 4	sm, atretic m	Deficient
	320	3	94 ± 6	42 ± 4	sm, m, ml	Deficient
Diabetic HCG	100	3	181 ± 16	32 ± 8	s, sm	Repair
	200	3	212 ± 10	44 ± 7	sm, m, ml, few l.	Hypertrophy
	400	3	307 ± 41	72 ± 7	sm, ml, & CL	Hypertrophy

* Standard error of the mean.
† Small follicles (s) measures up to 0.38 mm.; small medium (sm) 0.38–0.50 mm.; medium (m) 0.56–0.63 mm.; medium large (ml) 0.68–0.75 mm.; large (l) 0.81–1.0 mm. (CL) Corpora lutea.

tissue typically observed in the ovaries of diabetic rats (3). The administration of 320 μg. of FSH caused an increase in ovarian weight in the animals so treated. Study of the microscopic anatomy of these ovaries showed the presence of healthy, small, small-medium, medium follicles, and follicles which had reached medium-large size. As at the lower dose levels, no alteration was found in the morphological characteristics of the interstitial tissue. The average uterine weight of the injected rats (receiving 320 μg. of FSH) was slightly above that found in the non-treated diabetic group.

HCG-treated Diabetic rats: A total dose of 100 μg. of HCG did not have an effect on the ovarian weight of diabetic rats, but did elicit a definite increase in mean uterine weight, from 64 to 181 mg. (Table 1). Histological examination of the ovary disclosed healthy, small-medium follicles. The appearance of the interstitial tissue in these ovaries was greatly improved and almost restored to the normal condition; the nuclei were oval and vesicular, without the appreciable clumping or folding characteristic of the sub-normal ovarian interstitial tissue reported previously in untreated diabetic

rats (3). At this dose, however, the cytoplasm of the interstitial tissue still appeared pale in comparison to normal. At a total dose of 200 μg. of HCG an increase in mean ovarian weight from 21 mg. to 44 mg. was evident. An average uterine weight of 212 mg. was obtained in this group of treated rats from 64 mg. Furthermore, the dose of 200 μg. HCG caused full repair of hypertrophy of the ovarian interstitial tissue, and resulted in the growth of follicles attaining large size. Ovaries from this group also manifested medium-sized, fluid-filled, luteinized follicles containing degenerating eggs and many macrophagic cells. At the highest dose of HCG, 400 μg., a marked increase in ovarian weight occurred, the average weight being above that of normal, non-diabetic rats. Medium-large follicles, a few corpora lutea, and fully repaired interstitial tissue were present. The average uterine weight in diabetic rats receiving 400 μg. HCG was increased to 307 mg. from the average value of 64 mg. observed in untreated diabetic rats; this weight was quite comparable to the average (357 mg.) weight of uteri removed from non-diabetic normal rats.

DISCUSSION

In a study reported by Shipley and Danley (10) it was shown that the administration of relatively large doses of crude anterior pituitary extracts to fifty-days-old, alloxan-diabetic rats resulted in an ovarian weight increase comparable to that obtained by similar treatment of non-diabetic animals of the same age. From this observation they concluded that an imbalance in pituitary function, rather than ovarian insufficiency, is responsible for gonadal dysfunction in alloxan-induced diabetic rats.

In the present study, the administration of hypophyseal follicle stimulating hormone appeared to evoke an ovarian response that was essentially identical to that obtained by other workers following similar treatment of adult hypophysectomized rats of the same strain (9). The ovary of the adult diabetic rat thus appears to be as sensitive to FSH as is the ovary of adult hypophysectomized rats. The presence of healthy small follicles in the ovaries of the non-treated diabetic rat makes it unwise, however, to rule out the secretion of at least small amounts of gonadotrophic hormones by the pituitary gland of the diabetic rat, adding to the effectiveness of the exogenously administered FSH.

The administration of HCG resulted in full repair of the interstitial tissue in the diabetic rats studied. In addition, dose levels of 200 μg. HCG elicited the appearance of healthy, small-medium follicles and some luteinization of the theca interna. A total dose of 400 μg. of HCG produced medium-large follicles, extensive luteinization of follicles, and the appearance of corpora lutea in the ovaries of diabetic rats. Since follicular growth is not stimulated in the ovaries of hypophysectomized rats treated with ICSH (9), the ovarian follicle response in the treated diabetic rats

suggests stimulation of hypophyseal function, either directly by the HCG or indirectly by ovarian hormone output.

The ovarian response of the diabetic rat to exogenous administration of trophic hormones must also be considered in the light of the recent report that diabetic rat pituitary gonadotrophic hormone content is normal or even increased; differences in plasma titers were not detected, however (7). The results of the present study would thus lend support to the hypothesis that defective or insufficient release of hypophyseal gonadotrophic hormones is probably a major factor responsible for the impaired gonadal function observed in diabetic female rats.

REFERENCES

1. Reis, R. A., E. J. De Costa and M. D. Altweiss: *Amer. J. Obstet. and Gynec.* **60:** 1023. 1950.
2. Eastman, N. J.: *Obstet. Surv. Baltim.* **1:** 3. 1946.
3. Lawrence, A. M. and A. N. Contopoulos: *Acta Endocrinol.* In Press. 1960.
4. Davis, E. M., N. W. Fugo and K. G. Lawrence: *Proc. Soc. Exper. Biol. and Med.* **66:** 638. 1947.
5. Linden, J. A. and B. B. Longwell: *Proc. Soc. Exper. Biol. and Med.* **70:** 607. 1949.
6. Hultquist, G. T.: *Acta Path. Microbiol. Scand.* **27:** 695. 1950.
7. Lawrence, A. M., A. N. Contopoulos and M. E. Simpson: *Proc. Soc. Exper. Biol. and Med.* **99:** 35. 1958.
8. Nelson, N.: *J. Biol. Chem.* **153:** 375. 1944.
9. Wooten, E., M. M. Nelson, M. E. Simpson and H. M. Evans: *Endocrinology* **63:** 860. 1958.
10. Shipley, E. G. and K. S. Danley: *Amer. J. Physiol.* **150:** 84. 1947.
11. Jensen, H., M. E. Simpson, S. Tolksdorf and H. M. Evans: *Endocrinology* **25:** 57. 1939.
12. Evans, H. M., M. E. Simpson, S. Tolksdorf and H. Jensen: *Endocrinology* **25:** 529. 1939.

THE EFFECT OF HYPOTHALAMIC LESIONS ON THE TESTES AND PROSTATE OF MALE DOGS[1,2]

JULIAN M. DAVIDSON AND WILLIAM F. GANONG

Department of Physiology, School of Medicine, University of California, San Francisco, California

ABSTRACT

The effects of hypothalamic lesions on testicular function were studied in 21 mature male dogs. Destruction of the posterior median eminence (8 dogs) resulted in testicular and prostatic atrophy without evidence of adrenocortical or thyroid inhibition. In these dogs, seminiferous tubule diameter and testicular and prostatic weight and histology were similar to those of hypophysectomized dogs. One animal with a lesion involving the most caudal part of the posterior median eminence had atrophic seminiferous tubules and normal interstitial tissue. In 12 animals lesions did not involve the posterior median eminence, and testicular weight and histology were normal. Serial evaluation of sperm count and motility in 13 of the dogs with lesions revealed a prompt disappearance of motile sperms in four of the six animals with posterior median eminence lesions. In the other two dogs, spermatogenesis persisted for eight and 21 days respectively before declining to negligible levels. Four dogs with lesions in the anterior and middle median eminence showed a moderate decline in the number and motility of spermatozoa ejaculated, without atrophy of the gonads. Sperm count and motility remained normal in two of three dogs with normal testes and lesions sparing the median eminence. The significance of these findings is discussed.

TESTICULAR atrophy following hypothalamic lesions has been reported in the male dog (1, 2, 3), rat (4), and guinea pig (5), but detailed study of the effects of such lesions on the histology of the gonads and accessory reproductive organs are not available. In the present paper, the weight and histology of the testes and prostate and the quality and quantity of semen ejaculated for dogs with hypothalamic lesions are reported.

METHODS

Sixty-three sexually mature male dogs were studied. In eight of these animals, testicular biopsies were the criterion of sexual maturity and reproductive normality. In the remaining animals, the criterion was an ejaculate containing large numbers of motile sperms. Ejaculation was induced by manual massage of the penile bulb through

Received November 9, 1959.

[1] Supported by grants-in-aid from the Commonwealth Fund and the Research Committee Academic Senate, University of California.

[2] This data was submitted by the senior author in partial fulfillment of the requirements for the Ph.D. degree in Animal Physiology at the University of California. Present address: Dept. of Anatomy, University of California, Los Angeles.

its preputial covering. The semen was collected in graduated tubes and placed in a bath of warm water. The motility evaluation was made immediately after ejaculation by viewing a drop of semen placed on a warm slide under the microscope. Total sperm content per ejaculate was estimated by counting the sperm cells in a hemocytometer after making a 1/100 dilution of semen with 10% neutral formol in saline. The following arbitrary categories were used in evaluating motility: "A"; 75% or more of the spermatozoa in vigorous progressive motion. "B"; 50 to 75% of the spermatozoa in progressive motion. "C"; less than 50% in progressive motion. "D"; no motile spermatozoa. No animals were included in the experiments unless they showed large numbers of spermatozoa of "A" motility in two specimens collected two to three days apart in the preoperative period.

Electrolytic lesions were produced by the technique of Hume and Ganong (6) in 21 of these normal dogs. Six were subjected to hypophysectomy by the transbuccal approach and 12 were castrated. After a three-week rest period, urinary output was measured in nine of the dogs with lesions for at least two days (2), and thyroidal radioactive iodine uptake was determined (7). Seven dogs with lesions were anesthetized with pentobarbital and the right adrenal vein cannulated by the technique of Hume and Nelson (8). Adrenal venous blood samples were centrifuged immediately after collection and the plasma stored in the frozen state. 17-hydroxycorticoid content was determined by the method of Silber and Porter (9).

Except for one dog with a lesion that died 19 days postoperatively, all operated animals were sacrifieced 22–44 days postoperatively. The testes, prostates, adrenals and thyroids were removed, weighed, and fixed in Bouin's solution. The testes were weighed in 24 normal dogs and the prostate in 16. Representative sections of the testes were stained with hematoxylin and eosin, or with iron hematoxylin and aniline blue. The testes and prostates of three normal dogs were similarly prepared. Thyroids, adrenals and prostates were stained with hematoxylin and eosin. The pituitary was removed for subsequent bioassay in 9 dogs. The results of the assays will be reported separately. The hypothalamus in these dogs and the hypothalamus with attached pituitary in the others were removed, fixed in formalin, and serially sectioned at 16 μ. Every tenth section was stained with hematoxylin and eosin. The details of each lesion were reconstructed on a sagittal projection of the dog hypothalamus. In some animals, camera lucida tracings were made of the outlines of Leydig cell nuclei, and the areas of the nuclei measured by planimetry. Estimates of the mean diameter of the seminiferous tubules were made by using an eyepiece micrometer attachment to the microscope. Under suitable magnification, twenty roughly circular cross sections of seminiferous tubules were randomly selected in each testis studied. The smallest diameter of each tubular cross section was measured and recorded. The average of these 20 readings was the "mean tubular diameter," expressed in arbitrary micrometer units.

RESULTS

Marked testicular atrophy was present in 8 dogs with hypothalamic lesions. The area common to the lesions in these animals (Fig. 1) was in the infundibular stem and corresponded to the area designated posterior median eminence in Hume's classification of hypothalamic areas (3). No evidence of testicular atrophy was found in 12 dogs in which this region was spared (Fig. 1). The area destroyed by the lesions in these latter dogs included the anterior and middle part of the median eminence and much of the ventromedial and dorsomedial nuclei. The mean testicular weight of dogs with posterior median eminence lesions was 490 ± 70 mg./kg. body

weight.[3] In dogs with lesions elsewhere, it was 1250 ± 40 mg./kg., in 16 reproductively normal dogs, 1350 ± 60 mg./kg., and in six hypophysectomized dogs, 496 ± 40 mg./kg.

The histological picture of the testes and prostate in dogs with gonadal atrophy was essentially the same as that observed after hypophysectomy. The mean diameter of the seminiferous tubules in the dogs with posterior median eminence lesions was 16.0 ± 0.5 arbitrary units, and in dogs with

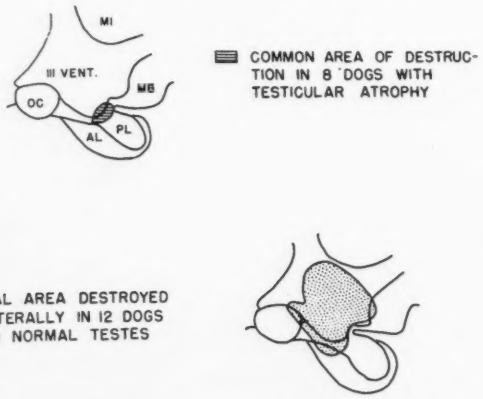


FIG. 1. Location of hypothalamic lesions associated with gonadal atrophy in male dogs. AL = Anterior lobe of pituitary. PL = Posterior lobe of pituitary. MI = Massa intermedia. MB = Mammillary bodies. III vent. = Third ventricle. OC = Optic chiasm.

lesions elsewhere 24.6 ± 0.6 units. In the hypophysectomized dogs, it was 15.6 ± 1.0 units. In the atrophied testes (Fig. 2) spermatozoa and spermatids were absent. Spermatocytes were drastically reduced in number, often being clumped together and presenting other unusual features. The lumina of the seminiferous tubles were obliterated.

There was severe involution of the interstitial tissue of the testis in the dogs with posterior median eminence lesions. The Leydig cells were reduced in size, their nuclei were pyknotic, and there was marked shrinking of the cytoplasm. In several dogs, planimeter measurements of the area of Leygid cell nuclei in the preoperative testicular biopsy specimen and the testicular specimen obtained at autopsy permitted objective verification of the effects of lesions on the interstitial cells. In two dogs with posterior median eminence lesions, the estimated decreases in Leydig cell nuclear area after lesion production were 38% and 34% respectively.

The prostate gland was studied in 13 dogs with lesions. Six of these dogs had testicular atrophy. The mean weight of the prostate in these animals was 315 ± 79 mg./kg. body weight as compared with a mean value of

[3] Mean $\pm$ standard error of the mean.

654 ± 71 mg./kg. for 24 normal dogs, and 530 ± 100 mg./kg. for the seven dogs with lesions and normal testes. In six hypophysectomized dogs the value was 289 ± 50 mg./kg., while in 12 castrate dogs, it was 258 ± 54 mg./kg. Histologically, the prostate in dogs with posterior median eminence lesions was severely atrophic with reduction of the glandular tissue to the thin lining of narrow clefts in a fibrous stroma (Fig. 3). These conditions were essentially identical to those found in hypophysectomized and castrate

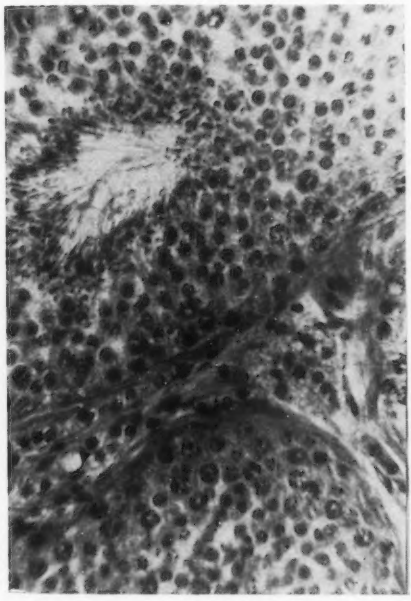


FIG. 2A. Preoperative biopsy specimen of dog testis (×240. Hematoxylin and eosin).

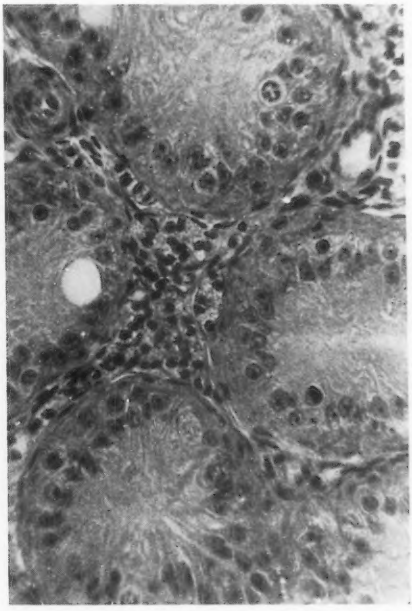


FIG. 2B. Testis of dog 28 days after production of hypothalamic lesion (×240. Hematoxylin and eosin).

animals. The histological findings in the dogs with lesions in other parts of the hypothalamus were not significantly different from that in normal dogs.

One dog with severe atrophy of the seminiferous tubules and a sharp decline in testicular weight showed interstitial tissue which was generally healthy in appearance. The area of Leydig cell nuclei in this dog showed an estimated increase of 9% between the preoperative biopsy and the autopsy specimen. The lesion involved the posterior limb of the infundibulum and part of the anterior, but at least a portion of the posterior median eminence was intact. The lesion extended into the anterior-most portion of the mammillary body and the caudal portion of the ventromedial nucleus.

Thirty-five ejaculates were collected from three unoperated control dogs over a period of 50 to 60 days. Sperm counts ranged from 86 million to 850

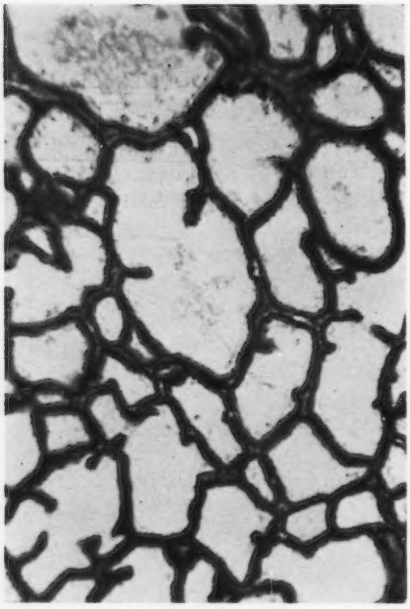


FIG. 3A. Normal dog prostate (×55. Hematoxylin and eosin).

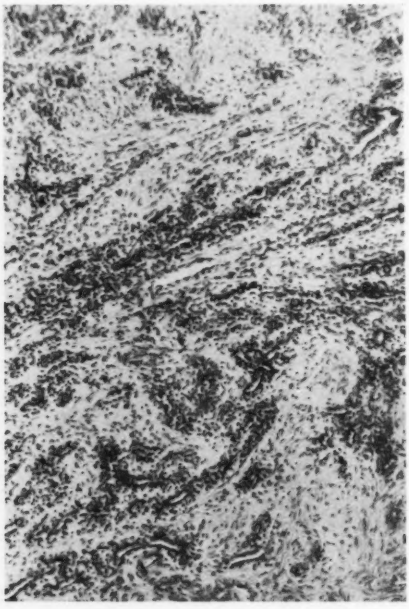


FIG. 3B. Prostate of dog 47 days after production of hypothalamic lesion (×55. Hematoxylin and eosin).

million per ejaculate. Sperm motility was rated "A" in all but three of these specimens, and in these three it was rated "B." In each of four hypophysectomized dogs, semen collections were attempted several times commencing three to five days postoperatively. One dog ejaculated 18 million sperms of "B" motility three days after hypophysectomy, but, except for this specimen, ejaculates could not be obtained in the hypophysectomized dogs.

The effects of diencephalic lesions on sperm count and motility were studied in 13 dogs. Two of these animals, one with a unilateral lesion in the thalamus and dorsal hypothalamus, and the other with a lesion above the median eminence, continued to produce ejaculates containng high sperm counts and with good motility. Testicular histology was normal in both dogs. In 4 other dogs without testicular atrophy, there was a deterioration of semen quality without aspermia. In one of these animals ejaculation ceased after lesion production and returned only shortly before autopsy. Another dog in this group produced only one specimen of low count and poor motility, but this animal had distemper in the postoperative period and was not studied consistently. The other dogs continued to ejaculate intermittently, and both sperm count and motility ratings were generally below

the normal control values. In all 4 of these animals, the lesions destroyed part of the anterior or middle median eminence, and in one case, there was unilateral destruction of the posterior median eminence.

In the remaining 7 dogs, ejaculation of semen containing motile sperm had ceased at the time of autopsy. One of these dogs had a small unilateral lesion above the median eminence and normal testes, but the remaining six had gonadal atrophy and posterior median eminence lesions. In this latter group of dogs, 4 animals showed characteristics essentially the same as that following hypophysectomy, i.e. prompt cessation of ejaculation. The remaining two dogs in this group produced ejaculates containing large numbers of motile sperm until the eighth and fifteenth postoperative days respectively. Thereafter, ejaculation declined, and finally ceased. Typical results in dogs with lesions are plotted in Figure 4.

In 6 normal dogs, the thyroid took up $30.2 \pm 3.7\%$ of a dose of I^{131} injected 48 hours previously. The iodine uptakes in 3 hypophysectomized dogs were 1.1%, 2.1%, and 1.6%. In all dogs with lesions I^{131} uptake was in the normal range, and thyroid histology was also essentially normal.

Mean left adrenal weight in the dogs with lesions and testicular atrophy was 51.3 ± 3.9 mg./kg., while in the dogs with lesions elsewhere, it was 53.1 ± 5.6 mg./kg. In 10 normal dogs, the value was 54.9 ± 0.5, and in the six hypophysectomized dogs the value was 33.3 ± 4.5. In all dogs with lesions in which it was measured, 17-hydroxycorticoid output after surgical trauma was within the previously published normal range for this laboratory (10). Urinary output was measured in five dogs with atrophy of the gonads and four without. Output was greater than 700 ml./day on two con-

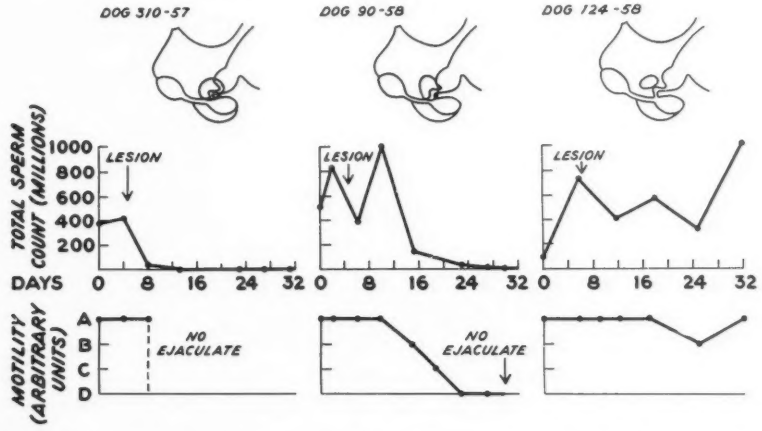


FIG. 4. Effect of hypothalamic lesions on sperm count and motility. See text for definition of motility units. Shaded areas in diagrams of hypothalamus represent extent of bilateral destruction.

secutive days in one of the former group and two of the latter. Mean output in normal dogs is 256 ±9.6 ml./day (2).

DISCUSSION

In the 8 dogs with testicular atrophy, there was a common area of destruction in the posterior median eminence. This finding is in agreement with previous studies on the male dog (1, 2, 3). Lesions in other parts of the median eminence were not effective.

The data permit comparison of the results of hypophysectomy with those of hypothalamic lesions on a quantitative basis by the criteria of testicular weight and tubular diameter, and qualitatively, by the criteria of testicular and prostatic histology. By all these parameters, the effects of posterior median eminence lesions appear to be identical to those of hypophysectomy. It seems probable, therefore, that there was inhibition of the release of FSH and ICSH in these animals. In another study (11), we have found that lesions in this location decrease the pituitary content of both FSH and ICSH. Apparently, therefore, both secretion and production of these hormones is depressed.

In ascribing testicular atrophy to the results of lesions in a hypothalamic area so closely related to the pituitary, it is necessary to consider the possibility that the gonadal atrophy is secondary to pituitary infarction. This possibility seems unlikely for a number of reasons. In the four cases in which the pituitary was examined histologically, it appeared normal. Acidophils and basophils were present in roughly normal proportions. At least 70% of the adenohypophysis must be removed before gonadal atrophy appears in the male dog (12). The effective lesions in the present series of dogs destroyed only the most caudal of the hypophyseal portal vessels, leaving the majority of them intact. Large lesions in the anterior and middle median eminence, destroying more of the portal vessels, did not lead to testicular atrophy. Furthermore, partial pituitary stalk section with the insertion of an impermeable barrier between the posterior median eminence and the pituitary in dogs has been found to cause a decrease in the basophils but no other change in pituitary cytology. Only with the final severance of all vessels of the portal system did atrophy and shrinkage of the adrenohypophysis occur in the dog (13). Finally, gonadal atrophy in the present study was not correlated with the extent of damage to the median eminence, but rather with destruction of a localized specific area.

The one animal which showed extensive atrophy of the seminiferous tubules with normal Leydig cells is of considerable interest. Unfortunately, the prostate was not examined in this animal. The lesion in this dog involved less of the median eminence than those producing diffuse gonadal atrophy. None of the other animals had a lesion in this precise location.

There is no data in the literature regarding selective inhibition of FSH secretion with hypothalamic lesions, but there is considerable evidence in favor of a center in the anterior hypothalamus concerned with the control of ICSH release. Thus, Dey (14) observed constant estrus and ovaries containing many unruptured follicles in guinea pigs following anterior hypothalamic lesions. Hillarp (15), Greer (16), and Van Dyke and associates (17) have confirmed this observation in the rat.

The studies of sperm count and motility are of considerable interest because they give a sequential picture of the changes in gonadotrophic and gonadal hormone secretion. Two of 6 dogs with lesions and gonadal atrophy showed spermatogenesis for a period of 8 to 13 days, followed by disappearance of ejaculation (Fig. 1). The lesions in these two animals were not demonstrably different from those in the four animals in which ejaculation did not occur after lesion production. The hypophysectomized dogs showed prompt cessation of ejaculation. Therefore, the reason for the "delayed response" in 2 dogs remains obscure. It may be relevant that in these 2 dogs, the preoperative sperm counts were higher than they were in any of the other dogs with posterior median eminence lesions. However, there are several reports in the literature of somewhat similar "delayed responses" in other endocrine phenomena following lesions. Greer (16) found that some rats that responded to hypothalamic lesions with persistent estrus did so only after several normal postoperative estrus cycles. Similarly, Holmes, Hughes, and Zuckerman (18) found that following pituitary stalk section, female monkeys showed one or two menstrual periods before becoming amenorrheic.

In the 4 dogs with damage to the anterior and middle portion of the median eminence, there was a decrease in semen quality without gonadal atrophy. This may well be due to a partial inhibition of gonadotrophin secretion, although no definite data are available on this point in the present study. The depression of spermatogenesis in one animal with a lesion above the median eminence remains unexplained.

It has been suggested that posterior pituitary hormones may be neurohumors involved in regulating gonadotrophin secretion (19). In this regard, it is interesting that there was no correlation between the presence or absence of diabetes insipidus and gonadal atrophy. Since vasopressin secretion is claimed to be largely under the control of the supraoptic nucleus while oxytocin secretion is regulated by the paraventricular nucleus, these experiments do not exclude a possible role for oxytocin in the regulation of FSH and ICSH secretion (20).

Acknowledgments

The authors are indebted to Angela Boryczka, Annette Lowe, Roy Shackleford, and Noreen Wells for technical assistance.

REFERENCES

1. GANONG, W. F., D. S. FREDERICKSON AND D. M. HUME: *Endocrinology* **57**: 355. 1955.
2. DAILY, W. J. R. AND W. F. GANONG: *Endocrinology* **62**: 442. 1958.
3. HUME, D. M.: in Reticular Formation of the Brain. Eds. H. H. Jasper, L. D. Proctor, R. S. Knighton, W. C. Knoshay and R. T. Costello. pp. 231–248. Little, Brown and Co., Boston. 1958.
4. MCCANN, S. M.: *Am. J. Physiol.* **175**: 13. 1953.
5. BOGDANOVE, E. M. AND S. A. D'ANGELO: *Endocrinology* **64**: 53. 1959.
6. HUME, D. M. AND W. F. GANONG: *J. Electroencephalog. and Clin. Neurophysiol.* **8**: 136. 1956.
7. FREDERICKSON, D. S., W. F. GANONG AND D. M. HUME: *Proc. Soc. Exper. Biol. and Med.* **89**: 416. 1955.
8. HUME, D. M. AND D. H. NELSON: *J. Clin. Endocrinol. and Metab.* **15**: 839. 1955.
9. SILBER, R. H. AND C. C. PORTER: *J. Biol. Chem.* **210**: 923. 1955.
10. GANONG, W. F.: in Comparative Endocrinology. Ed. A. Gorbman. John Wiley and Sons, New York. 1959.
11. DAVIDSON, J. M., A. N. CONTOPOULOS AND W. F. GANONG: Submitted to *Endocrinology*. 1959.
12. GANONG, W. F. AND D. M. HUME: *Endocrinology* **59**: 293. 1956.
13. ROTHBALLER, A.: Personal Communication. 1959.
14. DEY, F. L.: *Amer. J. Anat.* **69**: 61. 1941.
15. HILLARP, N. A.: *Acta Endocrinol.* **2**: 11. 1949.
16. GREER, M. A.: *Endocrinology* **53**: 380. 1953.
17. VAN DYKE, D. C., M. E. SIMPSON, S. LEPKOVSKY, A. A. KONEFF, J. R. BROBECK: *Proc. Soc. Exper. Biol. and Med.* **95**: 1. 1957.
18. HOLMES, R. L., E. BRODIE HUGHES AND S. ZUCKERMAN: *J. Endocrinol.* **18**: 305. 1959.
19. MARTINI, L., L. MIRA, A. PECILE AND S. SAITO: *J. Endocrinol.* **18**: 245. 1959.
20. OLIVECRONA, H.: *Acta Physiol. Scand.* Suppl. 136. 1957.

NOTES AND COMMENTS

EFFECT OF LIGHT ON THE WEIGHT OF THE PINEAL IN THE RAT[1]

ABSTRACT

Twenty-two day old male and female rats were placed in continuous light, almost continuous darkness or under usual day-night conditions, for six to twenty-five weeks. At autopsy it was found that the pineals of the animals housed under continuous light were significantly smaller than the pineals of either the laboratory controls or the rats kept in darkness. This suggests that the pineal may play a role in the changes in reproductive activity seen in rats exposed to continuous light.

Removal of the pineal in the immature rat is followed by an increase in ovarian weight (1, 2, 3), in the cockerel by a decrease in testicular weight (4). Administration of pineal extracts also effects gonad weight. Recent work indicates that the ovaries are smaller in immature rats given pineal extracts (5), while the gonads of cockerels are heavier following such treatment (4). Since exposure to altered light conditions also changes the weight of the gonads in rats (6, 7) it seemed that a study of the pineal in such animals might prove of interest.

METHODS

Fifty-three male and forty-seven female Sprague Dawley rats, twenty-two days of age, were divided into three groups and placed in continuous light, in a naturally lighted laboratory or in nearly constant total darkness. For brief periods each day the rats housed in darkness were exposed to a faint green light while being fed and cleaned. Two fluorescent lights provided the continuous light source. The intensity of the light in the cages was about seventy foot-candles.

The male rats were all autopsied nine and one half weeks after the initiation of the experiment while the females were autopsied at intervals varying from six to twenty-five weeks of treatment. The animals were killed quickly and the body weight taken. The pineal was dissected from the brain under a binocular microscope and immediately weighed on a Roller-Smith balance. The ventral prostates were weighed, and the ovaries were examined and weighed.

RESULTS

An analysis of variance was carried out on the data from the female rats of varying ages. (Table 1 first experiment.) The weights of the pineals from the rats in continuous darkness were not significantly different from the controls, but the pineals from the rats in continuous light were significantly lighter in weight ($P < .001$) than those of the other two groups. In a second experiment (Table 1) the decrease in pineal weight of rats in continuous light for 25 weeks was significant at $P < .001$ by t-test.

Analysis of the data for the male rats (Table 2) showed that the variance of the pineal weights of rats in continuous light was significantly smaller than that of the other two groups. Therefore, the Fisher-Behrens test was performed rather than an analysis of variance. Again, there was no significant difference between the pineal weights of rats in continuous darkness and the controls, but the pineal weights of the rats in continuous light were significantly lighter (P .001) than the other two groups. The differences in body weight between the three groups of male rats were not statistically significant.

Some of the male rats housed in darkness had not yet matured, as evidenced by the small size of the prostate in these animals. All of the males in the other two groups had

Received October 2, 1959.

[1] Supported by a grant (1556) from the National Institutes of Health, United States Public Health Service.

TABLE 1. BODY WEIGHTS OF FEMALE RATS KEPT IN CONSTANT LIGHT, TOTAL DARKNESS, OR UNDER USUAL LABORATORY CONDITIONS FOR SIX TO TWENTY-FIVE WEEKS

Length of exposure	Control rats			Rats in constant light			Rats in constant darkness		
	No. of rats	Body wt., gm.	Pineal wt., mg.	No. of rats	Body wt., gm.	Pineal wt., mg.	No. of rats	Body wt., gm.	Pineal wt., mg.
				First experiment					
6 weeks	3	202	1.6	3	175	1.1	3	208	1.7
7 weeks	4	212	1.9	6	198	1.3	5	195	1.4
8 weeks	2	212	1.4	3	187	1.1	3	227	1.3
9 weeks	3	236	1.6	3	195	1.1	2	257	1.3
25 weeks	2	277	1.5	3	255	0.5	2	321	1.5
Mean for all rats			1.6			1.1			1.5
				Second experiment					
25 weeks	8	286	1.5	12	283	1.0			

well-developed prostates. The ovaries of the female rats kept in continuous light for nine weeks or longer became increasingly follicular with time, rarely exhibiting anything but normal appearing or atretic follicles.

DISCUSSION

These results indicate that light directly, through the nervous system, or indirectly through hormone imbalance, affects the pineal body of the rat. The decrease in size of the pineal reported here may be related to changes in the secretory activity of the pituitary, gonads, thyroid or adrenal cortex, since, in rodents, all these glands seem to be affected by light (7, 8, 9, 10). The fact that rats, in persistent estrus due to their exposure to constant light, will exhibit normal appearing reproductive cycles when injected with pineal extracts (11) indicates that the change in the pineal reported here is more likely related to a shift in the secretion of the gonadotrophins than it is to changes in the secretion of the other hormones just mentioned. On the other hand, it may be associated with changes in the secretion of hormones involved in the maintenance of water balance. There is some evidence (12) that the antidiuretic hormone may be secreted more actively in rats under continuous light.

Acknowledgments

We are indebted to Dr. Mindel C. Sheps, Department of Preventive Medicine, Harvard Medical School, for advice on the statistical treatment and interpretation of the

TABLE 2. BODY AND ORGAN WEIGHTS OF MALE RATS KEPT IN CONSTANT LIGHT, TOTAL DARKNESS OR UNDER USUAL LABORATORY CONDITIONS FOR NINE AND ONE-HALF WEEKS

	No.	Body wt., gm.	Pineal wt., mean ±S.E., mg.
Control Rats	17	300	1.7 ±0.09
Rats in Constant Light	17	302	1.2 ±0.06
Rats in Total Darkness	19	277	1.7 ±0.11

data, and to Mrs. Elizabeth Moore, Biological Research Laboratories, Harvard School of Dental Medicine, for the computations.

VIRGINIA M. FISKE, G. KAREN BRYANT[2] AND JANET PUTNAM

Department of Zoology and Physiology
Wellesley College
Wellesley, Massachusetts

REFERENCES

1. IZAWA, Y.: *Tr. Soc. path. jap.* **16**: 72. 1926.
2. SIMONNET, H., L. THIEBLOT AND T. MELIK: *Ann. d'endocrinol.* **12**:202. 1951.
3. KITAY, J. I.: *Endocrinology* **54**: 114. 1954.
4. SHELLABARGER, C. J.: *Endocrinology* **51**: 152. 1952.
5. KITAY, J. I. AND M. D. ALTSCHULE: *Endocrinology* **55**: 782. 1954.
6. BROWMAN, L. G.: *J. Exper. Zool.* **75**: 375. 1937.
7. FISKE, V. M.: *Endocrinology* **29**: 187. 1941.
8. DEMPSEY, E. W. AND H. F. SEARLES: *Endocrinology* **32**: 119. 1943.
9. PUNTRIANO, G. AND J. MEITES: *J. Endocrinology* **48**: 217. 1951.
10. FORTIER, C.: *Rev. Canad. Biol.* **9**: 70. 1950.
11. JOCHLE, W.: *Endokrinologie* **33**: 287. 1956.
12. FISKE, V. M. AND R. O. GREEP: *Endocrinology* **64**: 175. 1959.

[2] Present address, Biological Research Laboratory, Harvard School of Dental Medicine, Boston, Massachusetts.

METABOLISM OF 16-KETOESTRONE BY HUMAN PLACENTA[1]

ABSTRACT

The *in vitro* conversion of 16-ketoestrone to compounds characterized as estriol and 16-ketoestradiol by human placenta has been described. The pathways for the formation of these two compounds by the placenta appear to be distinct, and an outline for the relationship of these 16-oxygenated estrogens in the human placenta is proposed.

Although 16-ketoestrone has been postulated as a natural precursor of estriol (1), its actual role in this regard has not yet been clearly defined. Slaunwhite and Sandberg isolated 16-ketoestrone from the urine of a subject given estrone (2), while Stimmel *et al.* had clearly demonstrated the *in vivo* conversion of 16-ketoestrone to estriol and 16-ketoestradiol[2] (1). Breuer *et al.* have reported the conversion of 16-ketoestrone to many 16-oxygenated metabolites by human liver and ovary *in vitro* (3). The present report describes the *in vitro* conversion of this compound by human placenta into substances with the characteristics of estriol and 16-ketoestradiol. Interestingly, the pathways for the formation of these two metabolites appear to be distinct.

MATERIALS AND METHODS

Term human placentas were obtained on delivery and processed according to methods previously reported (4). The 10,000×g. and 105,000×g. supernatant fractions were used. Erlenmeyer flasks containing tissue preparations, cofactors and substrate dissolved in propylene glycol were incubated with agitation at 37° C in air for one hour.

Details of the extraction procedure have been reported (4, 5). The incubation mixtures

Received November 5, 1959.

[1] Supported by grants from the National Institutes of Health (Grant No. A-2270) and the Josiah Macy, Jr. Foundation.

[2] 16-ketoestradiol as used herein refers to 16-ketoestradiol-17β.

TABLE 1. CONVERSION OF 16-KETOESTRONE TO ESTRIOL AND 16-KETOESTRADIOL

Substrate	μg. estrogens formed	
	Estriol	16-ketoestradiol
16-ketoestrone	13.3	9.0
	13.0	10.0
	13.4	12.5
	12.8	7.5
16-ketoestradiol	0	—

Conditions: Placental 10,000×g. supernatant fraction equivalent to 11 grams wet weight of tissue, 100 μg. of substrate and the following in μM per cc.: phosphate buffer pH 7, 50, niacinamide 40, ATP 10, and DPN 10 in a total volume of 5 cc. incubated for one hour in air at 37° C. The estrogens were purified as described in the text and isolated by elution from paper chromatograms.

were extracted three times with six volumes of $HCCl_3$ and the $HCCl_3$ taken to dryness. The $HCCl_3$ residues were subjected to a pentane/90% methanol partition to remove lipid, and the aqueous methanol extract was used for further purification. Phenolic separations were carried out as previously described (4), followed by paper chromatography and elution. Counter current distribution was carried out by techniques previously reported (6). Paper chromatography was performed utilizing the Bush method (7) with three modified solvent systems: ligroin:toluene (2:1)/70% methanol, toluene/75% methanol and ethyl acetate: toluene (9:1)/60% methanol. The Heftman procedure for paper chromatography was also employed (8). Compounds were detected on the Bush chromatograms by the Turnbull Blue reaction (9). Estrogens were measured in duplicate by the fluorescence method of Engel (6) and by the Kober reaction as modified by Brown (10). The Bachman reaction was carried out on paper as described by Axelrod (11), and a purified estriol fraction was subjected to the David reaction (12). The 16-ketoestrone was synthesized by Dr. M. Huffman and was chromatographically pure.[3]

RESULTS

The incubation of 16-ketoestrone with human placental preparations, ATP and DPN always resulted in the formation of two major metabolites with the characteristics of estriol and 16-ketoestradiol (Table 1). Since the incubation of 16-ketoestradiol under the same conditions did not result in the formation of estriol, it is felt that 16-ketoestradiol is not an intermediate in the placental system (Table 1). Control experiments incubated without substrate did not contain detectable amounts of steroids, and zero time extraction of the complete system revealed only unchanged 16-ketoestrone (Table 2). Controls were always run simultaneously with each incubation. In keeping with previously reported studies, 16-ketoestrone and 16-ketoestradiol had only a fraction of the fluorogenic potency of estriol (13). It was therefore possible to measure a net increase

TABLE 2. CONTROL EXPERIMENTS

System	μg. total estrogen formed	Metabolites identified by chromatography
Complete system, incubated	36	estriol, 16-ketoestradiol
Complete system, zero time	0	16-ketoestrone (substrate)
Minus substrate, incubated	0	0

Conditions: Placental 10,000×g. supernatant fraction equivalent to 11 grams wet weight of tissue, 100 μg. 16-ketoestrone and the following in μM per cc.: phosphate buffer pH 7, 50, niacinamide 40, ATP 10 and DPN 10 in a total volume of 5 cc. incubated for one hour in air at 37° C. The metabolites were isolated and characterized as described in the text. The above values are the average of 20 duplicate experiments within which there was quantitative and qualitative agreement. The use of the 105,000×g. supernatant fraction under similar conditions gave equivalent results.

[3] The 16-ketoestrone was generously supplied by Dr. M. N. Huffman.

in fluorescence after incubation and to demonstrate that this was due to the formation of estriol (Table 2).

The estriol produced was characterized as a: fluorogenic, Kober chromogenic phenolic substance with Rf values identical with known estriol run in parallel in the three modified Bush systems described above. It gave positive Bachman and David reactions. Counter current distribution in a 70% methanol/$HCCl_3$:CCl_4 (4:1) system gave a homogeneous curve consistent with estriol. The absorption curve of the Kober chromogen was identical with standard estriol.

The 16-ketoestradiol was characterized as a: fluorogenic, Kober chromogenic, phenolic substance with Rf values identical with standard 16-ketoestradiol run in parallel in the three modified Bush systems and the Heftman system. It gave a fluorescence reaction with the Bachman reagent similar to standard 16-ketoestradiol.

One other definite metabolite appeared in smaller amounts and had a chromatographic mobility similar to 16α-hydroxyestrone but could not be identified.

DISCUSSION

If 16-ketoestrone is a natural precursor of estriol as postulated (1), its metabolism by the human placenta would be important for an understanding of estriol biosynthesis during pregnancy. The present report demonstrates the *in vitro* conversion of this compound to metabolites with the characteristics of estriol and 16-ketoestradiol, in yields of 10–12% for each. Breuer *et al.* have reported the *in vitro* conversion of 16-ketoestrone to many 16-oxygenated steroids including estriol and 16-ketoestradiol by human liver and ovary (3). The same tissues will also convert 16-ketoestradiol to estriol (3). In the present study and other work previously published (5), 16-ketoestradiol is not significantly metabolized by the placental system and therefore is apparently not an intermediate for the conversion of 16-ketoestrone to estriol under these conditions. This would suggest that the conversion of 16-ketoestrone to estriol is mediated by a distinct 16-keto hydrogenase. In keeping with this, is the report by Langer *et al.* (14) of the conversion of 16-ketoestrone to 16-ketoestradiol but not to estriol with the purified placental 17β-ol

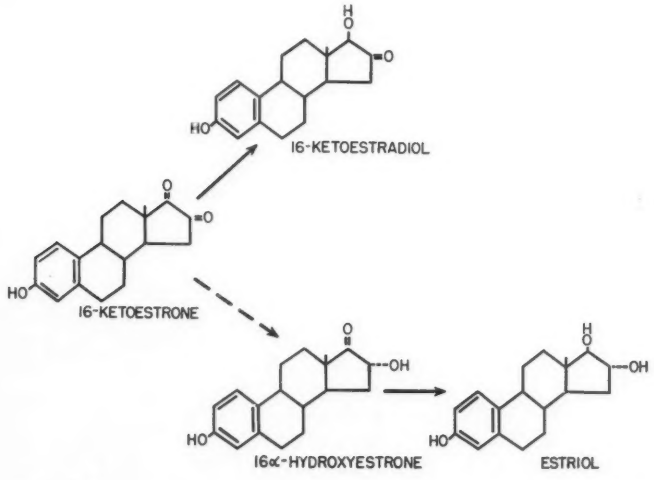


FIG. 1. Proposed pathway for the metabolism of C-16 oxygenated estrogens by human placenta.

dehydrogenase. Figure 1 demonstrates a proposed pathway for the metabolism of 16-oxygenated estrogens by the placenta. A likely intermediate between 16-ketoestrone and estriol is 16α-hydroxyestrone, since its rapid conversion to estriol by the human placenta has been demonstrated (5). Since the 105,000×g. supernatant fraction of placenta is active for all these reactions (Table 2), the postulated 16-keto hydrogenase would be a soluble enzyme. Preliminary studies with ammonium sulfate fractionation of the soluble placental preparations have not yet resulted in separation of the enzymatic activities.

KENNETH J. RYAN

Fearing Research Laboratory
Department of Obstetrics and Gynecology
Harvard Medical School
Boston, Massachusetts

Acknowledgments

The author wishes to express his appreciation to Miss P. Couch for technical assistance and to Dr. O. W. Smith for valuable criticism and advice.

REFERENCES

1. STIMMEL, B. F., A. GROLLMAN AND M. N. HUFFMAN.: *J. Biol. Chem.* **184:** 677. 1950.
2. SLAUNWHITE, W. R., JR. AND A. A. SANDBERG.: *Arch. Biochem.* **63:** 478. 1956.
3. BREUER, H., R. KNUPPEN AND G. PANGELS: *Acta Endocrinol.* **30:** 247. 1959.
4. RYAN, K. J.: *J. Biol. Chem.* **234:** 268. 1959.
5. RYAN, K. J.: *J. Biol. Chem.* **234:** 2006. 1959.
6. ENGEL, L. L., W. R. SLAUNWHITE, JR., P. CARTER AND I. T. NATHANSON: *J. Biol. Chem.* **185:** 255. 1950.
7. BUSH, I. E.: *Biochem. J.* **50:** 370. 1952.
8. HEFTMAN, E.: *Science* **111:** 571. 1950.
9. BARTON, G. M., R. S. EVANS AND J. A. F. GARDNER: *Nature* **170:** 249. 1952.
10. BROWN, J. B.: *Biochem. J.* **60:** 185. 1955.
11. AXELROD, L. R.: *J. Biol. Chem.* **201:** 59. 1953.
12. DAVID, K.: *Acta Brevia Neerl. Physiol. Pharmacol. Microbiol.* **4:** 64. 1934.
13. SLAUNWHITE, W. R., JR., L. L. ENGEL, P. C. OLMSTED AND P. CARTER.: *J. Biol. Chem.* **191:** 627. 1951.
14. LANGER, L. J., J. A. ALEXANDER AND L. L. ENGEL: *J. Biol. Chem.* **234:** 2609. 1959

EFFECT OF INTRAVENOUS o,p′ DDD ON PLASMA 17-HYDROXY-CORTICOID SECRETION IN THE DOG

ABSTRACT

Intravenous administration of 2,2-bis (2-chlorophenyl-4-chlorophenyl)-1,1-dichloroethane (o,p′ DDD) produced a marked decrease in the secretion rate of plasma 17-hydroxycorticoids of the dog when given in daily doses of 50–54 mg./kg. for 3 days or 75–95 mg./kg. for 2 days. A suitable infusion medium was prepared by rapid mixing of an ethanol, propylene glycol solution of o,p′ DDD with a commercial oil-in-water emulsion (Lipomul I.V.).

The development of a suitable vehicle for the intravenous administration of fat-soluble compounds offers a means of eliminating the problem of incomplete absorption frequently associated with the oral administration of compounds such as o,p′ DDD.[1]

Received November 9, 1959.

[1] 2,2-bis(2-chlorophenyl-4-chlorophenyl)-1,1-dichloroethane.

Moreover, the possibility of increased effectiveness of a smaller dose given for a shorter period of time presents opportunities for testing compounds in limited supply.

This paper describes the preparation of such an infusion medium together with data showing the effectiveness of o,p′ DDD in reducing the output of adrenal vein plasma steroids when the drug was given intravenously in this vehicle.

MATERIALS AND METHODS

Stock solutions of 5 to 15% o,p′ DDD were prepared by the addition of crystalline o,p′ DDD to a heated mixture of propylene glycol-absolute ethanol. The stock solution was then dripped slowly into a commercially available oil-in-water emulsion (Lipomul I.V.) while the emulsion was being shaken rapidly by machine. A vehicle containing 5 or 10 mg. o,p′ DDD per ml. was prepared in this manner by adding 10 ml. of either the 5% or 10% stock solution (equal parts propylene glycol-absolute ethanol) to 90 ml. Lipomul. Propylene glycol and absolute ethanol (1:2) were used to prepare the 15% stock solution of o,p′ DDD and 10 ml. of this concentrate was added to 90 ml. Lipomul for preparation of an emulsion containing 15 mg. o,p′ DDD per ml.

Adult male and female mongrel dogs ranging in weight from 90 to 20 kg. were used in these experiments. Various dose levels of the o,p′ DDD emulsion described above were infused daily via the cephalic vein. At 20 to 24 hours after the last infusion, anesthesia was induced with intravenous Nembutal (27 mg./kg. body weight). The right adrenal vein was cannulated using the technique of Hume and Nelson (1). Plasma from 10 minute samples of adrenal vein blood was used for steroid assay. Peterson's modification (2) of the Silber-Porter method was used for the determination of plasma 17-hydroxycorticoids.

RESULTS

Results are shown in Table 1. Plasma 17-hydroxycorticoid secretion rates in those animals treated with vehicle only (No. 1, 2, and 3) were within the range for untreated normal dogs. A marked decrease in 17-hydroxycorticoid output was seen after intravenous administration of o,p′ DDD (No. 4–8). With one exception, the animals were under Nembutal anesthesia while receiving the infusion. Dog No. 8 received the emulsion by rapid injection without anesthesia. It will be noted that o,p′ DDD in daily doses of 50 mg./kg. for 3 days or 75–95 mg./kg. daily for 2 days effectively inhibited 17-hydroxycorticoid secretion.

Variable results were obtained when combinations of 50 mg. o,p′ DDD/kg. were given on the first day followed by lower doses on the second and third day of treatment. In several dogs treated in this manner, plasma 17-hydroxycorticoids were markedly suppressed in some instances and normal in others.

In order to check the effect of the vehicle on plasma steroid content, recoveries of added hydrocortisone were made from samples of dog plasma together with samples containing the vehicle (Lipomul-propylene glycol-ethanol 90:5:5) mixed with dog plasma in concentrations approximating immediate post-infusion blood levels of the emulsion. There was no effect of vehicle on recovery of plasma 17-hydroxycorticoids.

TABLE 1. INHIBITION OF 17-HYDROXYCORTICOID OUTPUT AFTER INTRAVENOUS ADMINISTRATION OF o,p′ DDD

Dog No.	Sex	Wt. (kg.)	Concentration o,p′ DDD (mg./ml.)†	Daily dose o,p′ DDD (mg./kg.)	Infusion rate (ml./min.)	17-OH corticoids (plasma) (mcgm./min.)
1	♀	19.5	Vehicle only	None	4–5	5.4, 5.6, 7.2
2	♂	13.4	Vehicle only	None	4–5	10.1, 10.5, 11.0
3	♂	13.2	Vehicle only	None	3–5	7.3, 7.8, 7.6
4	♀	10.0	5	50	4–5	0.3, 0.3, 0.2
5	♂	16.2	5	54	4–5	0.5, 0.6
6	♀	13.3	5	50	4–5	0.5, 0.5, 0.5
7	♂	10.5	10	95	2–3	0.2, 0.2
8	♂	11.5	15	75	20	0.3, 0.3

† Dogs No. 1 through 6 received infusions daily for 3 days; dogs 7 and 8, for 2 days.

Although o,p′ DDD has been found to inhibit adrenocortical function when given orally (3, 4), this report demonstrates the effectiveness of intravenous administration of the drug based on the preparation of a vehicle which is adequate for intravenous use.

Acknowledgment

The invaluable assistance of Mr. Donald L. Barber is gratefully acknowledged.

WILLIAM W. TULLNER AND ROY HERTZ

Endocrinology Branch
National Cancer Institute
National Institutes of Health
Bethesda, Maryland

REFERENCES

1. HUME, D. M. AND D. H. NELSON: *Surgical Forum* **5**: 568. 1955.
2. PETERSON, R. E., J. B. WYNGAARDEN, S. L. GUERRA, B. B. BRODIE AND J. J. BUNIM: *J. Clin. Invest.* **34**: 1779. 1955.
3. CUETO, C. AND J. H. U. BROWN: *Endocrinology* **62**: 334. 1958.
4. VILAR, O. AND W. W. TULLNER: *Endocrinology* **65**: 80. 1959.

ANNOUNCEMENTS

GORDON RESEARCH CONFERENCE

CHEMISTRY, PHYSIOLOGY AND STRUCTURE OF BONES AND TEETH

11–15 July 1960
Kimball Union Academy
Meriden, New Hampshire

B. B. MIGICOVSKY, Chairman
W. P. NORRIS, Vice Chairman

11 July

Selected Communications

CLAYTON RICH, Chairman

W. P. L. MYERS and W. LAWRENCE, JR. Studies on the Influence of Cortisone on Serum Calcium Homeostasis

C. A. L. BASSETT. Factors Contributing to Osteogenesis *in vitro*

T. W. SPECKMAN and W. P. NORRIS. Variations in Retention Kinetics of Bone Seeking Isotopes

J. SAMACHSON and H. SPENCER. Comparison of Single and Multiple Doses of Sr^{85} and Ca^{45} in Man

J. M. JONES, P. J. KELLY and L. F. A. PETERSON. The Effect of Berylium on Bone, a Morphologic Study of the Progressive Changes in Rabbit Bone

Bone Induction

LEROY LAVINE, Chairman

J. S. NICHOLAS. Induction and Its Relationship to Bone Structure

M. Moss. Osteogenic Induction Factors

12 July

J. J. PRITCHARD. Recruitment of Osteoblasts Relating to Bone Induction

HANS SELYE. Induction of Bone by Tissue Scaffoldings

Nature and Mechanism of Hard Tissue Destruction

REIDAR F. SOGNNAES, Chairman

N. M. HANCOX. The Osteoclast

13 July

CHARLES M. DOWSE and WILLIAM NEUMAN. Metabolism of Bone Cells

GEORGE NICHOLS, JR., NANCY NICHOLS, ANDRE B. BORLE, STEIN SCHARTUM, GILBERT VAES. Cellular Metabolism of Bone

PAUL GOLDHABER. Further Observations on Experimental Bone Resorption in Tissue Culture

Organic Matrix in Calcification

M. J. GLIMCHER, Chairman

F. PAUTARD. The Nature of the Organic Matrix and Its Role in the Development of Calcified Structures

14 July

L. JOHNSON. Histochemical Changes in Organic Matrix Accompanying Calcification

A. SOBEL. Role of the Organic Matrix in the Nucleation and Growth of Bone Crystals

Calcium Metabolism

F. MCLEAN, Chairman

J. Vincent. Metabolic Bone at the Histological Level

15 July

Calcium Metabolism

H. F. DELUCA, Chairman

A. M. SHANES. Movement of Calcium in Non-skeletal Tissues

M. R. URIST. Calcium and Protein Relations in the Blood

R. H. WASSERMAN. Passage of Calcium Across Biological Membranes

Attendance. Requests for attendance at the conference, or for additional information, should be addressed to W. George Parks, Director, Department of Chemistry, University of Rhode Island, Kingston, Rhode Island. From 13 June to 2 September 1960 mail should be addressed to Colby Junior College, New London, New Hampshire.

Registration and Reservations. Attendance at the Conference is by application. Individuals interested in attending the conference are requested to send their applications to the Director at least two months prior to the date of the Conference. *All applications must be submitted on the standard application form which may be obtained by writing to the office of the Director.*

PRELIMINARY ANNOUNCEMENT OF AN INTERNATIONAL SYMPOSIUM TO BE HELD AT PURDUE UNIVERSITY, LAFAYETTE, INDIANA ON JUNE 16, 17, AND 18, 1960

GROWTH; MOLECULE, CELL, AND ORGANISM

THURSDAY, JUNE 16, 1960

F. L. HOVDE, President, Purdue University
Welcoming Address

F. H. C. CRICK, University of Cambridge, Cambridge, England
Macromolecules and Natural Selection

M. B. HOAGLAND, Massachusetts General Hospital, Boston, Massachusetts
Chemical Mechanism in Protein Synthesis

D. MAZIA, Department of Zoology, University of California, Berkeley, California
The Plan of Cellular Reproduction

T. T. Puck, University of Colorado, Denver, Colorado
Aspects of Mammalian Cell Growth in Tissue Culture

Friday, June 17, 1960

A. A. Moscona, University of Chicago, Chicago, Illinois
Studies on Tissue Reconstruction from Dissociated Cells
M. Sussman, Brandeis University, Waltham, Massachusetts
Cellular Differentiation in the Slime Mold
J. Brachet, Universite Libre de Bruxelles, Bruxelles, Belgium
The Role of Ribonucleic Acid and -SH Groups in Morphogenesis
C. W. Wardlaw, University of Manchester, Manchester, England
Investigation of Floral Morphogenesis
A. C. Braun, The Rockefeller Institute, New York City, New York
The Plant Tumor Problem and Its Relation to Neoplastic Growth
M. Singer, Cornell University, Ithaca, New York
The Role of the Wound Epithelium in Vertebrate Regeneration

Evening

James Bonner, California Institute of Technology, Pasadena, California
The Biology of Plant Growth

Saturday, June 18, 1960

Animal Section

C. M. Williams, Harvard University, Cambridge, Massachusetts
Insect Metamorphosis: An Approach to the Study of Growth
T. H. Jukes, American Cyanimid Co., Stamford, Conn.
Vitamins, Antibiotics, and Growth
V. A. Drill, G. D. Searle & Co., Chicago, Illinois
Steroids and Growth
J. Hammond, University of Cambridge, Cambridge, England
Growth in Size and Body Proportions of Farm Animals
E. Knobil, Harvard Medical School, Boston Mass.
Endocrine Regulation of Growth; The Pituitary Growth Hormone
G. Pincus, Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts
Senescence

Microbial Section

H. Ephrussi-Taylor, Labratoire de Genetique Physiologique, Gif-Sur Yvette, France
Control of Synthetic Functions through Transforming Principles
S. S. Cohen, University of Pennsylvania, Philadelphia, Pennsylvania
Virus-Induced Acquisition of Metabolic Function
A. B. Pardee, University of California, Berkeley, California
Enzyme Feedback, Repression and Regulation of Metabolism
E. Zeuthen, Carlsberg Foundation, Copenhagen, Denmark
Studies on Growth and Division in the Protozoan Cell
A. Novick, University of Oregon, Eugene, Oregon
Bacteria with High Specific Enzyme Levels
H. O. Halvorson, University of Illinois, Urbana, Illinois
Bacterial Sporogenesis

Plant and Soils Section (Morning)

H. JENNY, University of California, Berkeley, California
Plant Root-Soil Interactions
P. DANSEREAU, University of Montreal, Montreal, Canada
The Origin and Growth of Plant Communities
F. C. STEWARD, Cornell University, Ithaca, New York
Some Problems of Cells: Their Growth and Nutrition

Plant Section (Afternoon)

F. W. WENT, Missouri Botanic Gardens, St. Louis, Missouri
Light and Temperature Effects on Plant Growth
G. S. POUND, University of Wisconsin, Madison, Wisconsin
Growth Aspects of Plant Virus Infections
G. E. BLACKMAN, University of Oxford, Oxford, England
The Analysis of Plant Responses in the Vegetative Phase to Environmental Factors

Soils Section (Afternoon)

A. G. NORMAN, University of Michigan, Ann Arbor, Michigan
The Biological Environment of Roots
C. E. MARSHALL, University of Missouri, Columbia, Missouri
Colloid Chemistry of the Soil in Relation to Plant Nutrition
M. B. RUSSELL, University of Illinois, Urbana, Illinois
Soil Physical Conditions Affecting Plant Growth

Evening

ANCEL KEYS, University of Minnesota, Minneapolis, Minnesota
Changes with Aging

For additional information, write: Dr. M. X. Zarrow, Department of Biological Sciences, Life Science Building, Purdue University, Lafayette, Indiana.

INTER-SOCIETY CYTOLOGY COUNCIL

Inter-Society Cytology Council, Annual Scientific Meeting, Palmer House, Chicago, Illinois, September 23, 24, 25, 1960; Paul A. Younge, M.D., Secretary, 1101 Beacon Street, Brookline 46, Massachusetts.

PROGRAM

EIGHTH MICROCIRCULATORY CONFERENCE

APRIL 12, 1960

EAST ROOM—STATLER-HILTON HOTEL, NEW YORK 1, NEW YORK

8–9:00 A.M.—Registration
9:00 A.M.—Welcome by Chairman of Microcirculatory Conference
JOHN W. IRWIN, Massachusetts Eye and Ear Infirmary, Boston
9:05 A.M.—A tribute to Normand L. Hoerr
by EDWARD H. BLOCH, Western Reserve University
9:10 A.M.— CIRCULATION OF THE PITUITARY
Chairman: ROY O. GREEP, Harvard University

General and comparative aspects of pituitary circulation
JOHN D. GREEN, University of California Medical Center, Los Angeles
Relationships of pituitary vasculature to physiology of the pars distalis
JOHN W. EVERETT, Duke University
Initial Discussant: JOHN D. GREEN, University of California Medical Center, Los Angeles

10:30 A.M.—Coffee

Studies on the small blood vessels of the living pituitary
W. CURTIS WORTHINGTON, JR., Medical College of South Carolina
Initial Discussant: J. DEGROOT, University of California Medical Center, Los Angeles
Electron microscopic studies on the capillaries of the anterior pituitary gland
MARILYN G. FARQUHAR, The Rockefeller Institute
Initial Discussant: SANFORD L. PALAY, National Institutes of Health

2:00 P.M.— CIRCULATION OF BONE MARROW

Chairman: ALBERT S. GORDON, Washington Square College

Experimental investigation of microcirculation in bone marrow
P-I BRÅNEMARK, University of Lund
Initial Discussant: RIOJUN KINOSITA, City of Hope Medical Center
Electron microscopic study of the vascular bed of bone marrow
L. M. ZAMBONI and DANIEL C. PEASE, University of California Medical Center, Los Angeles
Initial Discussant: LEON WEISS, Harvard University
The effect of environment on proliferation and maturation of nucleated red cells
ALLAN J. ERSLEV, The Jefferson Medical College
Initial Discussant: O. P. JONES, University of Buffalo

4:00 P.M.—Business Meeting—HERBERT J. BERMAN, Secretary-Treasurer
4:15 P.M.—High Tea

THE NATIONAL VITAMIN FOUNDATION, INC.

Friday, October 7, 1960—Jewish Hospital of St. Louis, St. Louis, Missouri: An all-day Symposium on The Genetic Control of Biochemical Processes in Diseases of Metabolism, under the joint sponsorship of the Foundation, the Jewish Hospital of St. Louis, St. Louis University School of Medicine and Washington University School of Medicine. For further information write William Rubin, Director of Public Information, The National Vitamin Foundation, Inc.

CORRECTION

On line 6 of the Endocrinology Study Section announcement appearing on page 147 of the January 1960 issue of ENDOCRINOLOGY, the source of the available follicle-stimulating hormone reads "bovine" whereas it should read, ovine.